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KINETICS, MECHANISM AND INTIMATE RELATION BETWEEN CYCLOOXYGENASE AND PEROXIDASE ACTIVITIES OF PROSTAGLANDIN ENDOPEROXIDE SYNTHASE

by

MARICA BAKOVIC ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

DEPARTMENT OF CHEMISTRY

EDMONTON, ALBERTA
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THE UNIVERSITY OF ALBERTA
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The undersigned certify that they have read, and recommend to the faculty of Graduate Studies and Research for acceptance, a thesis entitled " Kinetics, Mechanism and Intimate Relation between Cyclooxygenase and Peroxidase Activities of Prostaglandin Endoperoxide Synthase" submitted by Marica Bakovic in partial fulfilment of the requirement for the degree of Doctor of Philosophy in Chemistry.

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Dedicated to my daughter, Lena,
my son Luka, and my husband Vasilije

ABSTRACT

Prostaglandin endoperoxide synthase is an integral membrane hemoprotein whose function is to catalyze the conversion of a membrane fatty acid, arachidonic acid, to prostaglandin H_2, a precursor of other prostaglandins and thromboxanes. The catalytic reaction for the following process has two general steps: (1) two molecules of oxygen are incorporated in arachidonic acid to form a transient endoperoxide hydroperoxide prostaglandin G_2 (the cyclooxygenase reaction) and (2) prostaglandin G_2 is reduced to an endoperoxide hydroxide, prostaglandin H_2 (the peroxidase reaction). To get more insight into the catalytic mechanism, the peroxidase and the cyclooxygenase functions of prostaglandin endoperoxide synthase are studied independently and a mutual relationship is established.

The peroxidase reaction was studied by using the steady-state method of initial rate measurements. It is found that the structurally related natural products, *p*-coumaric, ferulic and caffeic acids, serve as good reducing substrates of the enzyme higher oxidation states and support the peroxidase reaction initiated with hydrogen peroxide. The prostaglandin endoperoxide synthase-peroxidase reaction of the monophenols *p*-coumaric and ferulic acids obeys an irreversible ping-pong mechanism as does the horseradish peroxidase reaction. The hydroperoxide oxidizes Fe^{III} of the native enzyme to the $Fe^{IV} = O$ porphyrin π cation radical (compound I). Compound I is subsequently reduced by one molecule of the reducing substrate to compound II ($Fe^{IV} = O$). Finally, compound II is reduced by the other molecule of the reducing substrate to the native enzyme. A catechol, caffeic acid, is a good reducing substrate for the higher oxidation states of prostaglandin endoperoxide synthase but is also capable of binding to the native enzyme, and thus, competitively inhibiting the reaction of the native enzyme and hydrogen peroxide.

An interdependent relationship between the peroxidase and the cyclooxygenase activity is found to exist. By following the rates of disappearance of arachidonic acid and ferulic acid in a mixture of the two substrates, a constant 1:2 stoichiometric ratio between them is found. This indicates that arachidonic acid reacts with one enzyme intermediate, (compound I) and ferulic acid with two enzyme intermediates (the compound I-tyrosyl radical and compound II).

The oxygenase reaction of 11,14-*cis,cis*-eicosadienoic acid is studied in both the presence and the absence of Trolox C, yet another peroxidase reducing substrate. The rate of fatty acid product formation is twice that of the rate of the oxygen consumption. A mechanism for the spontaneous oxygenase reaction is proposed which indicates that the fatty acid reduces compound I by one-hydrogen atom transfer and the compound I-tyrosyl radical by one-oxygen atom transfer which lead to the formation of two molecules of fatty acid hydroxide. Trolox C prevents the enzyme inactivation by reducing an unproductive compound I-protein radical and compound II but does not affect the stoichiometry of the oxygenase reaction.

The pre-steady state kinetic study and numerical simulation of the oxygenase and the cyclooxygenase reactions unambiguously show that compound I reacts with the fatty acid in a bimolecular fashion which provides strong evidence against the tyrosyl-radical hypothesis (Dietz et al., (1987) *Eur. J. Biochem*, **166,** 173-180). According to the tyrosyl-radical hypothesis compound I is initially converted by an inter-molecular electron transfer to the compound I-tyrosyl radical which then reacts with the fatty acid which opposes existing evidence.

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CHAPTER ONE

PROSTAGLANDIN ENDOPEROXIDE SYNTHASE, AN IMPORTANT ENZYME IN THE ARACHIDONIC ACID CASCADE

1.1. INTRODUCTION

Arachidonic acid (20:4)* and other polyunsaturated fatty acids containing three to five cis methiline-interrupted double bonds are metabolically oxidized to an array of biologically active compounds generally known as eicosanoids. These compounds have been implicated as critical mediators in many diseases. Consequently, the enzymes that form these oxidative derivatives have received considerable attention, particularly as targets for novel pharmacological agents.

Three major catalytic pathways of the oxidative metabolism of 20:4 known as the arachidonic acid cascade include (i) the PGH synthase pathway to prostanoid compounds (PGH2, PGD_2, PGE_2, PGI_2 and $PGF_{2\alpha}$, and TXA_2), (ii) the lipoxygenase pathway to leukotriene and lipoxin compounds, and (iii) the cytochrome P-450 pathway to epoxy- and mono- and di-hydroxy eicosatrienoic acids. The focus here is on the PGH synthase catalytic pathway. This review begins with the biochemical and pharmacological significance of eicosanoids in the regulation of cell processes. Following this is a general overview of the regulation, structure, and catalytic properties of PGH synthase.

---

* Abbreviations: 20:4, arachidonic acid; PGH synthase, prostaglandin endoperoxide synthase; PGH_2, PGI_2 and $PGF_{2\alpha}$, prostaglandins H_2, D_2, E_2, I_2 and $F_{2\alpha}$; TXA_2, thromboxane A_2 (the subscripts to prostanoid nomenclature refer to the two double bonds in the structure); HETE-hydroxy eicosatetraenoic acid; G-protein, guanine nucleotide regulatory protein; PC, phosphatidyl choline; PE, phosphatidyl ethanol amine; GTP, guanosine tri phosphate; NSAIDs, nonsteroidal antiinflammatory drugs; DDC, diethyldithiocarbamate; PPHP, 5-phenyl-4-pentenyl hydroperoxide.

1.2. PHYSIOLOGICAL FUNCTIONS OF ARACHIDONIC ACID METABOLITES

Eicosanoids (prostaglandins, thromboxanes and leukotrienes) act as local hormones. Eicosanoids do not accumulate to any appreciable extent within cells but are synthesized upon demand. This is in contrast to peptide hormones and neurotransmitters which are usually stored in intracellular secretory vesicles and released in response to stimuli (1-3). Eicosanoids have been implicated as modulators of many physiological processes such as platelet aggregation, smooth muscle contraction, sleep-wake cycle, body temperature, pain, vascular secretion, gastric acid secretion, chemotaxis, and luteolisis. They are also immunoregulators and mediators of inflammatory diseases such as bronchial asthma, arthritis and psoriasis and renal and cardiovascular diseases (4-9).

Eicosanoids are synthesized in a manner specific to each cell and a wide array of chemical and mechanical stimuli can provoke their formation in cells (10-14). Blood platelets form mainly TXA_2 (5,15), smooth muscle cells produce PGI_2, and vascular endothelial cells produce PGI_2 and PGE_2 (5,16). Leukotrienes are synthesized primarily by airways mast cells, eosinophils and macrophages (16) and $PGF_{2\alpha}$ by bone marrow, adrenal glands (17) and ovaries (5).

Structurally similar eicosanoids can exert counteracting effects. Balance in the action of one group of compounds compared to the action of the other group is crucial for normal cell functioning. As examples, TXA_2 is a platelet aggregating agent, broncho- and vasoconstrictor, while PGI_2 inhibits platelet aggregation and broncho- and vasoconstriction (5,18); PGI_2 and PGE_2 act in an inhibitory manner but LTB_4 stimulates lymphocyte proliferation (5); and, PGD_2 and PGE_2 elicit opposite effects on the sleep-wake cycle and body temperature (9).

Eicosanoids exerts their physiological effects via G protein-linked receptors. Only recently some specific eicosanoid receptors have been characterized but much remains to be learned about the related second messenger systems (17). Multiple subtypes of PGE_2 receptors (EP_1, EP_2 and EP_3) are found which indicates diversity of the post-receptor signal transduction systems and cellular responses to this particular prostaglandin (17). Thanks to the discovery of specific agonists, molecular and biochemical differences of the three subtypes are well understood. PGE_2 affects the function of the receptor operated Ca^{2+} channels via EP_1, and the level of adenylate cyclase and GTP via EP_2 and EP_3 receptors (17-22).

TXA_2 and PGH_2 have the same pharmacological properties in many tissues and they share a common TXA_2 /PGH_2 receptor (23). The TXA_2 /PGH_2 receptor from human platelets has been purified (24) and cloned (25). TXA_2 /PGH_2 receptor belongs to G protein-coupled rhodopsin type receptor and might be linked to two different signal tranduction systems, i.e., the inhibition of adenylate cyclase and the stimulation of phospholipase C.

Members of the other families of the oxidative arachidonic acid metabolites are also physiologically active. Some epoxy and di-HETE derivatives from the cytochrome P-450 pathway affect Ca^{2+} mobilization, stimulate peptide hormones release and can inhibit Na^+/K^+ ATPase and PGH synthase (15). Primarily eicosanoids acts as proinflammatory agents and therefore, the regulation of their production is of great interest. Rapid growth of this research area will lead to better recognition in the near future concerning the role of eicosanoids in physiological and pathophysiological processes.

1.3. PGH SYNTHASE CATALYTIC PATHWAY

As outlined in Figure 1.1 the PGH synthase pathway includes three steps: (i) the hydrolysis of arachidonic acid from phospholipid esters by phospholipase A_2; (ii) the cyclooxygenation of free arachidonic acid to a transient

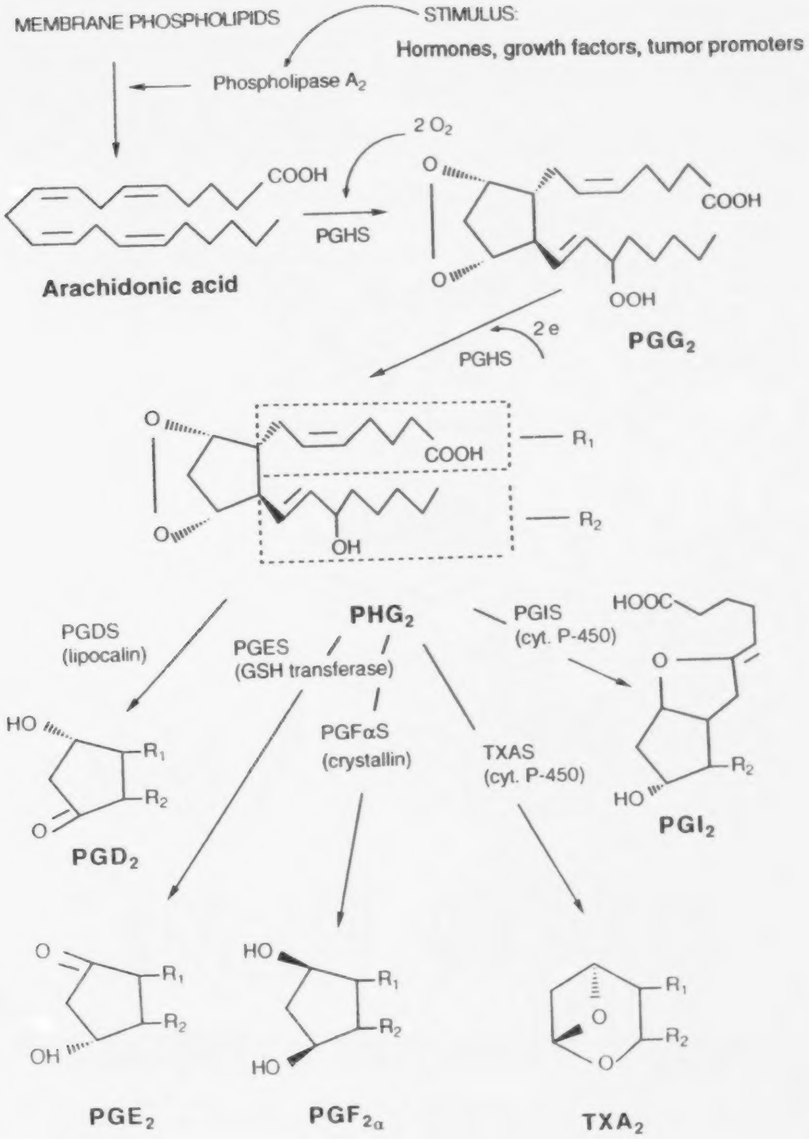

MEMBRANE PHOSPHOLIPIDS
STIMULUS:
Hormones, growth factors, tumor promoters
Phospholipase A2
2 O2
COOH
PGHS
Arachidonic acid
COOH
OOH
PGG2
2 e
PGHS
COOH
R1
R2
OH
PHG2
PGDS
(lipocalin)
PGES
(GSH transferase)
PGFαS
(crystallin)
TXAS
(cyt. P-450)
PGIS
(cyt. P-450)
HOOC
PGD2
PGI2
PGE2
PGF2α
TXA2

Figure 1.1. PGH synthase biosynthetic pathway for the formation of prostanoid compounds. PGHS, PGDS, PGES, PGF$_{\alpha}$S and PGIS correspond to prostaglandin H_2, D_2, E_2, $F_{2\alpha}$, and I_2 synthase and TXA_2S corresponds to thromboxane A_2 synthase. In the brackets are the structurally related enzymes.

endoperoxide hydroperoxide, PGG_2 and the reduction of PGG_2 to endoperoxide PGH_2 by PGH synthase, and (iii) the formation of a series of prostanoid compounds (PGH_2, PGD_2, PGE_2, PGI_2 and $PGF_{2\alpha}$, and TXAs) from PGH_2. Depending on cell type and function prostanoids are formed from PGH_2 through specific isomerization and reduction reactions by the action of a set of specific synthases (PGD, PGE, PGF_{α}, PGI and TXA synthases) (4,6,7,15).

1.3.1. Release of Arachidonic Acid

Availability of 20:4 is considered as a rate controlling step in prostanoid synthesis. Release of 20:4 occurs selectively and no other fatty acids from phospholipids are mobilized at the same time (26,27). It is not known which specific phospholipid is a direct precursor of 20:4 used in the PGH synthase pathway (4).

Initiation of the event occurs when a stimulus (hormones, growth factors) interacts with a specific receptor on the cell surface and activates the lipase system. The actual mechanism of this activation is not fully understood. It seems it includes G protein-linked mobilization of intracellular Ca^{2+} (26). Phospholipase A_2 may be the key enzyme for the hydrolysis of 20:4 from phospholipids. Two distinct isoforms, PLA_2-I and PLA_2-II, have been characterized. Both require Ca^{2+} for full enzymatic activity. Secretory PLA_2-I is structurally and functionally related to cobra venom and it is formed as a zymogen in the pancreas; PLA_2-II is a membrane bound form (28). PLA_2-I exists as a free form in platelets and cytosol (29,30) but also, was found as a peripheral membrane protein (31). Similar to other esterases, phospholipase A_2 amino acid residues Asp^{99}-His^{48} form a proton relay system which helps remove a proton from bound water and thus increases water nucleophilicity. The nucleophile OH^- attacks the phospholipid ester bond and releases 20:4. The Ca^{2+} ion has an important function to polarize the phospholipid carbonyl group, to stabilize the enzyme-phospholipid complex, and to bind free 20:4 until the fatty acid diffuses from the active site (28,32).

Other lipases such as phospholipase C and diacylglycerol lipase acting sequentially can also release 20:4 from membranes (26). However, the major sources of 20:4 are PC and PE, the substrates for phospholipase A_2. It is uncertain which type, membrane free or membrane bound phospholipase A_2, is involved in catalysis (28). More evidence is accumulating that it is the membrane-bound form. A large 100 kDa membrane protein isolated from a kidney is the most prominent candidate to be the 20:4-specific lipase (33,34).

The release of 20:4 appeared to be inhibited by the corticosteroid lipocortin (35). However, it is shown that corticosteroids suppress PGH synthase messenger RNA levels, but do not inhibit phospholipase A_2 (36,37). Thus the problem of control of the prostanoid synthesis at the level of fatty acid release remains open. Highly specific phospholipase A_2 inhibitors have yet to be found. A phospholipase A_2 activating protein has been discovered. This protein participates in the phospholipase A_2 binding to the membrane and thus can be a target for the inhibition of the 20:4 release and prostanoid synthesis (38).

1.3.2. PGH Synthase Isoforms and Regulation of Their Synthesis

As shown in Figure 1.1 PGH synthase is a bifunctional enzyme which exhibits the cyclooxygenase activity catalyzing PGG_2 formation and peroxidase activity which catalyzes a two-electron reduction of PGG_2 to PGH_2. Both activities are associated with a single protein of 600 amino acids and require heme (Fe^{3+}-protoporphyrin) as a prosthetic group. The purified enzyme is a homodimer of 70 kDa subunits that contain 3.5% carbohydrate of the mannose type (39-41). This PGH synthase was purified from bovine and ovine microsomes and represents an integral membrane protein , PGHS-1, which is very well characterized (42). This PGH synthase isoform was used in our kinetic and mechanistic study. Recently an inducible, transient form of PGH synthase, PGHS-2, has been found and characterized (42-48).

The two PGH synthase isoforms differ in their regulation and tissue distribution. Two distinct genes encode PGHS-1 and PGHS-2 (49-51). The PGHS-1 gene is relatively ubiquitously expressed in most tissues. It is linked to basal synthesis of prostanoids during cell differentiation and it is not suppressed by corticosteroid hormones (42). The PGHS-2 gene is highly restricted in cell types and tissues and is dramatically regulated by proinflammatory stimuli (mitogens, cytokines and growth factors) (52-55). These stimuli regulate PGHS-2 transcription through direct activation of protein kinase C and subsequent phosphorylation events. Dexamethasone inhibits PGHS-2 protein synthesis (56).

The existence of the two pools of PGH synthase was confirmed at the protein (57) and mRNA levels (58). Recently, it has been found that PGHS-1 can also be slightly induced and can also contribute to the up-regulation of prostanoid synthesis during monocytic differentiation (59). Unexpectedly, it was found that PGHS-1 mRNA and PGHS-2 mRNA are expressed equally in most human tissues except that PGHS-2 mRNA has a higher level in lung tissue (58).

Transcriptional regulation of the PGHS-2 gene involves 5'-flanking DNA sequences (60). A nuclear protein C/EBPβ binds specifically to PGHS-2 DNA elements and represents the central step in the regulation of PGHS-2 gene transcription. The functional relevance and precise sites of the interactions between the protein and DNA need to be established (61).

The discovery that the expression of PGHS-2 is highly inducible and selective, suggests that this isoform may be of particular importance in pathological processes such as inflammation and mitogenesis (62).

Conventional inhibitors have little selectivity for PGHS-1 and PGHS-2 (63) and the development of isoform specific inhibitors should enable the determination of the relative contribution of both isoforms in mediation of the pathological responses and normal cell functioning.

1.3.3. Important Residues and General Structure of PGH Synthase

Primary structures of PGHS-1 deduced from respective ovine, murine and human complementary DNA are highly (90%) similar. The important structural features of PGHS-1 are the heme-binding domain, the active site tyrosine, the aspirin acetylation site, glycosylation sites and membrane and epidermal growth factor domains (63-66).

From site-directed mutagenesis studies His^{309} has been proposed as the fifth (proximal) iron ligand, His^{388} as the distal residue and His^{207} as a group forming a salt bridge with one of the heme carboxy side chains. His^{388} is essential for both peroxidase and cyclooxygenase activities and His^{386} for peroxidase activity (67).

Replacement of Tyr^{385} with Phe^{385} selectively eliminates cyclooxygenase activity of PGH synthase (68). A tyrosyl radical generated during the PGH synthase reaction with PGG_2 has been previously identified by ESR (69) and was suggested as a key residue in the initiation step of the cyclooxygenase reaction.

Ser^{530} has been identified as the aspirin acetylation site. When Ser^{530} is acetylated, access of fatty acid to the cyclooxygenase site is blocked but there is no effect on the peroxidase reaction (51,65,66,70).

In native PGHS-1 there is a single protease sensitive region located near Arg^{253}. Binding of heme and NSAIDs make the enzyme resistant to proteolysis (71,72). PGHS-1 also contains three Asn-linked mannose type oligosaccharide groups (73). Both PGHS-1 and PGHS-2 are N-glycosylated at Asn^{68}, Asn^{144} and Asn^{410} and PGHS-2 is additionally glycosylated at Asn^{580} (74).

Trp^{387} is not essential for catalysis by PGHS-1 (75). Leu^{359} and Leu^{501} were expected to have an influence on dimer formation (75). Their mutation caused a partial or complete loss of cyclooxygenase activity but did not affect the dimer formation.

Amino acid sequences of PGHS-2 are 60% similar to PGHS-1 (51,76,77). The main differences between the isoforms exists in the hydrophobic signal-peptide region and carboxyl terminal region. The structural and functional domains of PGHS-1 are highly conserved in PGHS-2 indicating that an identical catalytic mechanism is expected for both isoforms (51,64-68,76,77).

The three dimensional structure of PGHS-1 has been recently resolved at 3.5 Å resolution (78). The asymmetric crystal unit contains two identical subunits with one heme per subunit. The overall spatial orientation of the active site of PGHS-1 has a high level of similarity with the active site of canine myeloperoxidase but also to two other heme containing peroxidases, yeast cytochrome c peroxidase and fungal lignin peroxidase. PGHS-1 is a globular protein with high (40%) helical content and almost without β-pleated sheets. Three folding domains were characterized; a catalytic domain, a membrane binding domain and an epidermal growth factor domain. The catalytic domain contains heme and two adjacent, but distinct peroxidase (PGG_2 and peroxidase reducing substrate) and cyclooxygenase (20:4 and NSAIDs) binding sites. The heme prosthetic group is located in a shallow cleft with two narrow grooves being suggested as two separate peroxidase binding sites. The proximal iron ligand is His^{388}. His^{207} and Glu^{203} are located on the distal side at a distance of 5 Å from the heme. The cyclooxygenase binding site represents a long narrow hydrophobic channel extended from the membrane binding domain to the central heme. This binding site contains Tyr^{385} near the edge of the heme and 10 Å from the iron. Ser^{385} is also located in the hydrophobic channel.

The X-ray crystal structure (78) reveals that there are no trans-membrane regions in PGHS-1 contrary to what has been suggested previously (64,65,74,79). The PGHS-1 dimer is associated with the membrane lipid surface through a large hydrophobic region allowing a direct entry of fatty acid from the lipid bilayer into the cyclooxygenase channel.

1.3.4. Requirement of Heme for Catalysis

PGH synthase is a hemoprotein and the heme prosthetic group is required for both peroxidase and cyclooxygenase activity. As in peroxidases the iron ion of the heme prosthetic group in the native PGH synthase is in its ferric, high-spin state and preferentially five-coordinate (80) to allow the peroxidase reaction. The hydroperoxide PGG_2 must enter the inner coordination sphere of iron in order to accept two electrons and to be reduced to PGH_2. At low temperatures the high-spin heme iron partially transforms to the low-spin heme iron and some non-specifically bound heme iron was identified by MCD and EPR spectroscopy (69,81).

Based on an analogy between the electronic spectra of PGH synthase and horseradish peroxidase, a histidine residue as the fifth iron ligand was initially suggested (82). As an alternative, a tyrosine residue as the axial ligand of the heme iron has been suggested based on the shape of the EPR tyrosyl radical signal and the chemical modification of the PGH synthase tyrosine residues with N-acetyl imidazole (80,83). There is a high degree of homology in the active site sequences of PGH synthase and thyroid- and myeloperoxidase, both having a histidine residue as the fifth iron ligand (84), which indicated that a histidine residue is probably the best candidate to be the fifth iron ligand. Only recently it has been shown that the low spin iron is coordinated to two imidazoles originating from two histidines (85), and the x-ray crystal structure finally confirmed that the His^{388} residue is the fifth iron ligand in PGH synthase (78).

After reaction with organic peroxide, PPHP, two distinct spectral intermediates of PGH synthase were obtained resembling compound I and compound II peroxidase intermediates (Figure 1.2) (82). Fe^{III} of the native enzyme is oxidized with the peroxide and an Fe^{IV}=O porphyrin π cation radical (compound I) is formed. The Soret spectrum of compound I has the same maximum (410 nm) but significantly lower intensity than the spectrum of the native enzyme. Compound I also has a characteristic featureless visible spectrum (not shown). This is an extremely unstable intermediate formed in the ms range at low temperatures (Figure 1.2A). Compound I is converted slowly to compound II. The Soret maximum of compound II appears to occur at 420 nm (Figure 1.2B) and two distinct absorption maxima are present in the visible region at 527 and 557 nm (not shown). The oxidation state of PGH synthase-compound II can be assigned either to the peroxidase Fe^{IV}=O species (86), produced by one-electron reduction of compound I, or to a Fe^{IV}=O-tyrosyl radical species as in the ES compound of cytochrome c peroxidase (87), produced by an intra-molecular one-electron transfer from a tyrosyl residue of the protein to the porphyrin π-cation radical. The spectral properties of PGH synthase during the course of the peroxidase and the cyclooxygenase reactions are similar indicating that the same intermediates could be involved in the two specific reactions (88). How these intra- and inter-molecular one-electron transfers are related is a crucial question in the catalytic mechanism and the main subject of this work.

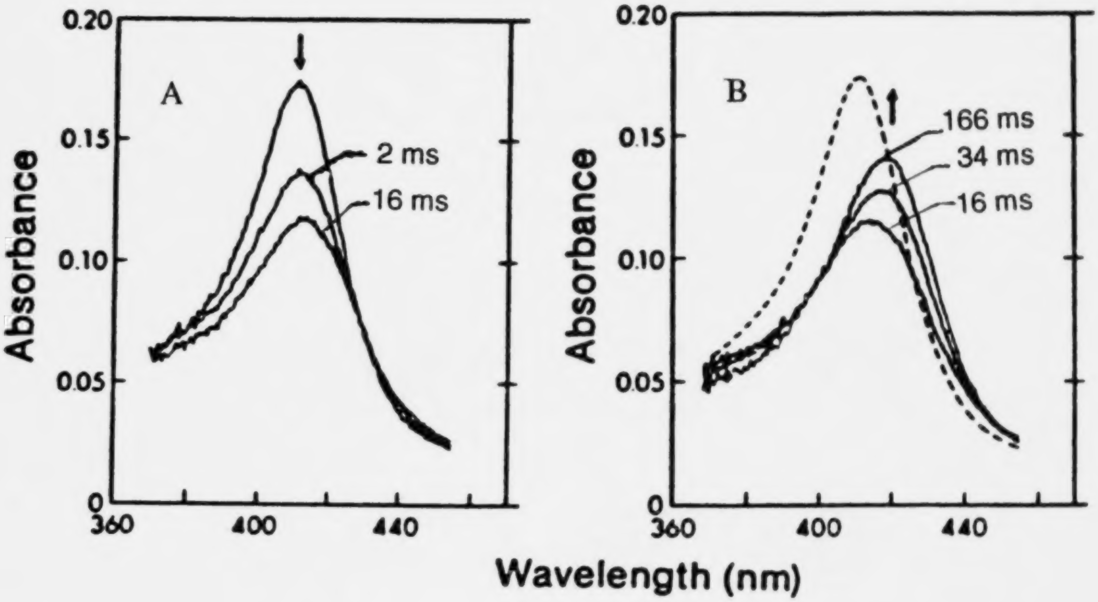


Figure 1.2 Formation of PGH synthase compound I (A) and compound II (B). PGH synthase was reacted with 5-phenyl-4-pentenyl hydroperoxide (A). Compound I was transformed to compound II in the absence of exogenous reducing substrate (B). (Addapted from reference 82 with permission)

1.3.5. The Cyclooxygenase Reaction and Mechanism

The chemical mechanism of the cyclooxygenase reaction has been known for almost three decades (89-91). Isotope labeling studies showed that oxygen incorporates at C_9, C_{11} and C_{15} of 20:4 (Figure 1.1) and that the removal of a hydrogen atom (probably *pro*-S-hydrogen atom from C_{13}) represents the initial rate limiting step in the cyclooxygenase reaction. Both products, PGG_2 and PGH_2, were isolated and identified (92,93). The carbon-centered 20:4 radical was detected by the spin-trapping method (94,95) but it is not proven at which carbon atom the odd electron is actually located. It is generally assumed (96) that it is C_{13} that loses a hydrogen atom initially and then isomerizes to the C_{11} radical. The catalyzed oxygenation reaction resembles the autooxidation reaction in many ways. The oxygen-insertion steps are believed to proceed spontaneously with no significant enzyme participation. After the C_{11} radical is formed, oxygen attacks the radical resulting in the C_{11}-hydroperoxyl radical formation. This radical attacks the double bond at C_9 and after cyclization forms another (C_{15}) radical. The new radical reacts with the second molecule of oxygen at C_{15} and forms the hydroperoxide endoperoxide radical $PGG_2\bullet$. After a hydrogen atom addition from the enzyme to the $PGG_2\bullet$ radical, the hydroperoxide endoperoxide PGG_2 is formed in the final step of the reaction.

The aliphatic side chains in PGG_2, PGH_2 and other prostanoids (Figure 1.1) are predominantly in the *trans* position suggesting that the enzyme specifically controls the conformation of 20:4. Appleton and Brown (97) proposed that PGH synthase fixes the position of the C_9-C_{10} single bond of 20:4 and holds the fatty acid molecule in a kink conformation before the hydrogen atom is abstracted. In addition, the enzyme probably keeps the endoperoxide hydroperoxide radical $PGG_2\bullet$ in a proper orientation and thus prevents eventual side reactions (98). Finally, the principal role of the enzyme is to abstract the hydrogen atom from the fatty acid in the initiation phase and to donate the hydrogen atom in the final phase of the cyclooxygenase reaction when the enzyme does not generally change the oxidation state.

There is considerable evidence that PGH synthase needs to be activated before it initiates the oxygenation of 20:4 and that a hydroperoxide is required (99-103). The polarographic measurement of the oxygen consumption is a standard procedure for the determination of the cyclooxygenase activity and the role of hydroperoxide in the initiation of the cyclooxygenase reaction. An induction (lag) phase appears to occur in oxygen consumption traces, indicating that during the initial period there is an insufficient amount of hydroperoxide to activate the reaction. Furthermore, the lag phase and the overall oxygen consumption rate are affected either by (i) changing the initial concentration of hydroperoxide (usually present as an impurity in 20:4 preparations) (ii) introducing peroxidase electron donors (they prevent the enzyme inactivation), or by (iii) interfering with 20:4 binding (this is a basis for the inhibitory action of NSAIDs) (99-103).

In the absence of peroxidase electron donors a tyrosyl radical was formed and the Soret spectrum of compound II concomitantly appeared (104,105). The detection of the tyrosyl radical is considered as key evidence for the reaction mechanism in which the compound-I tyrosyl radical species abstracts a hydrogen atom from 20:4 and thus initiates the oxygenation. After fast addition of two molecules of oxygen to the fatty acid carbon radical, the $PGG_2\cdot$ radical is formed. According to this hypothesis, compound II is the enzyme intermediate which donates the hydrogen atom to the endoperoxide hydroperoxide radical $PGG_2\cdot$ in the final step of the reaction. PGG_2 is formed and the compound I-tyrosyl radical is regenerated. The main consequence from this reaction mechanism is that the compound I-tyrosyl radical can be regenerated many times, implying a branched-chain free radical reaction which leads to the accumulation of PGG_2. Results will be presented in this thesis that oppose the branched-chain tyrosyl radical hypothesis.

The role of the tyrosyl radical in the reaction cycle is a complicated and controversial issue (106). It is evident that the tyrosyl radical is formed during the peroxidase reaction between the hydroperoxide and native enzyme and during the peroxidase-cyclooxygenase reaction between the hydroperoxide, 20:4 and the enzyme (104,105). Tyr^{385} is essential for catalysis (68) and it is located in the fatty acid binding site in the vicinity of the heme (78). There are two principal tyrosyl radical signals detected by ESR, a doublet formed initially and a narrow singlet formed at longer times (105,107, 108). The appearance of the singlet radical correlated with enzyme inactivation not with enzyme activity (108). The addition of a peroxidase electron donor (phenol) prevents the appearance of the doublet and suppresses the appearance of the singlet tyrosyl radical signal but does not inhibit the cyclooxygenase reaction (108). It has been suggested that the two tyrosyl radicals originate from different tyrosine residues and that at least the doublet can be competent in catalysis (107,109). However, there is evidence that the tyrosyl radical signals are from a single residue which casts doubt on the hypothesis that the source of the doublet is competent in catalysis (110). Furthermore, in the Mn^{3+}-PGH synthase catalyzed cyclooxygenase reaction there are no tyrosyl radicals but some unknown singlet protein radicals were detected (108). The manganese enzyme also needs hydroperoxide for the activation of the cyclooxygenase activity (111,112). Which enzyme transient initiates the reaction has not been established but the Mn^{IV}-protein radical has been suggested (112), similar to the compound I-tyrosyl radical for the iron enzyme.

The evidence presented suggests that the tyrosyl radical could be involved in a peroxidase-dependent suicide inactivation of the enzyme. PGH synthase is very prone to lose heme and thus both activities. Even during enzyme purification a stabilizing agent ought to be present to prevent the heme destruction (39-41, 88,96,106). Some amino acid residues, among them tyrosine residues, could be cooxidized during the inactivation but they have not been identified (113). This should not be a serious problem with modern amino-acid sequence techniques but could provide very useful information about the residues involved in the inactivation mechanism.

1.3.6. The Peroxidase Reaction and Mechanism

The peroxidase function of PGH synthase can be studied separately from the cyclooxygenase function by initiating the reaction with an organic or lipid hydroperoxide and some reducing substrate other than fatty acid. Two general reactions of PGH synthase peroxidase are the electron transfer and the oxygen transfer reactions. The electron transfer is the most common mechanism and occurs by two sequential electron transfers (89). The oxygen transfer has been detected with alkylaryl sulfides (114) but a detailed mechanism for this reaction is yet to be determined. There is also evidence for a mixed one- and two-electron transfer for the PGH synthase reducing substrate acetaminophen but also no details about the mechanism are known (115).

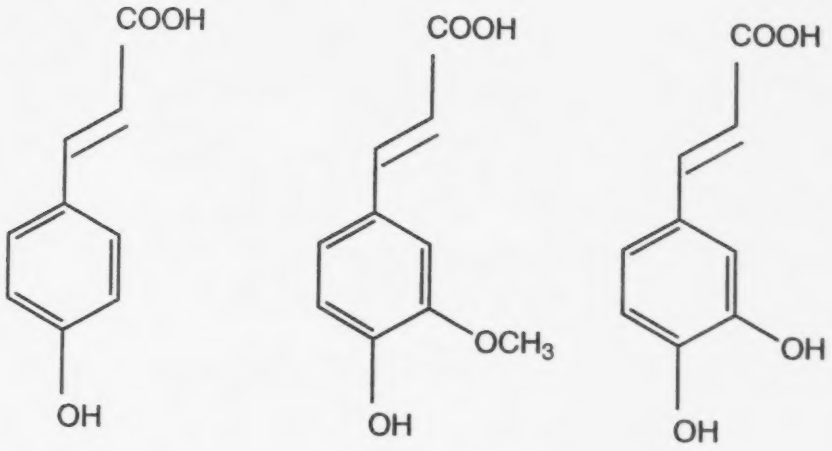
The one-electron transfer mechanism is a classical peroxidase mechanism characteristic for horseradish peroxidase, myeloperoxidase and other peroxidases. The peroxidase mechanism consists of a two-electron oxidation of the native enzyme by a peroxide resulting in the formation of compound I and the corresponding alcohol, and the two one-electron reductions of compound I to compound II and to the native enzyme by some electron (hydrogen atom) donor.

Substrate free radicals formed during these two steps diffuse from the enzyme into bulk solution where they combine to form dimers and/or other stable products.

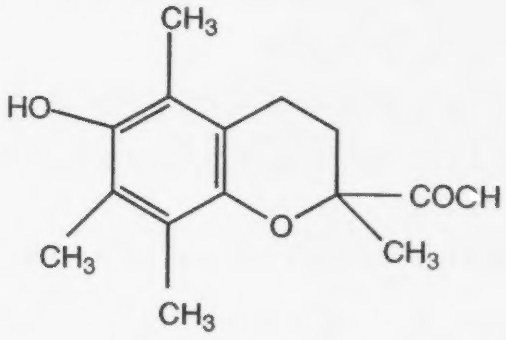
PGH synthase is very active toward lipid hydroperoxides including PGG_2 and 15-HPETE as well as an organic hydroperoxide PPHP (40,82). It is less active toward small molecules such as H_2O_2 (a typical substrate for horseradish peroxidase) or bulk molecules such as cumene hydroperoxide (a typical substrate for cytochrome P-450) (40). This suggests that the hydroperoxide binding site of PGH synthase is of intermediate size in comparison to the binding sites of horseradish peroxidase and cytochrome P-450 (40,96). The rate constants for the first step in the peroxidase reaction were determined for PGG_2 and PPHP under transient state conditions (82, 104) and for PGG_2 and 15-HPETE under steady-state conditions (116, 117).

A short life of compound I and a rapid peroxidase-dependent inactivation of the enzyme has limited more extensive kinetic and mechanistic studies on this enzyme. Limited information was provided from the steady state experiments because these experiments have been performed with classical spectroscopic methods on the time scales when the inactivation was significant. The investigation of the single elementary steps by transient state kinetics was limited to low temperatures and to the reaction of the native enzyme and compound II. A detailed picture about the compound I (the key enzyme intermediate in catalysis) reaction with peroxidase reducing substrates remains unknown.

Some of the best electron donors of PGH synthase are polyhydroxylated compounds, phenols and aromatic diamines (118). Some compounds known as good substrates for classical peroxidases (NADH, aromatic amines , iodide) are poor substrates for the PGH synthase peroxidase (118). We will show that a


COOH
COOH
COOH
OCH3
OH
OH
OH
OH

p-Coumaric Acid   Ferulic Acid   Caffeic Acid


CH3
HO
COCH
O
CH3
CH3
CH3

Trolox C

Figure 1.3 PGH synthase reducing substrates used in this study

series of naturally occurring cinnamate derivatives, *p*-coumaric, ferulic and caffeic acids, and vitamin E related compound, Trolox C, (Figure 1.3) serve as good reducing substrates for the PGH synthase peroxidase. These compounds possess suitable chromophores for the spectroscopic measurements. We exploited this substrate property in a detailed kinetic and mechanistic study of the peroxidase reaction and its relationship with the cyclooxygenase reaction of PGH synthase.

The enzyme undergoes rapid inactivation by hydroperoxide and 20:4 in the absence of the stabilizing agent DDC (119,120). It has been shown (120-122) that DDC protects the enzyme from bleaching and loss of activity by its ability to serve as a peroxidase reducing substrate. The kinetic rate constant for the reaction of compound II with DDC was precisely determined (122). This provided for the first time a possibility to quantitatively evaluate the kinetic parameters for the reaction of compound II with other reducing substrates such as ascorbic acid (123), phenol and hydroquinone (124) in the presence of DDC i.e., under conditions when no enzyme inhibition interfered with kinetic measurements. In addition, this investigation indicated that the compound I-tyrosyl radical is not formed in the presence of sufficient amount of reducing substrate.

There is long standing evidence that different peroxidase electron donors can cooxidize with 20:4 (125-132) but a detailed mechanism of the simultaneous peroxidase-cyclooxygenase reaction remained unclear. Peroxidase electron donors affect the oxidation of 20:4 in two ways: they stimulate the cyclooxygenase reaction at lower concentrations and inhibit at higher concentrations (133). The protection from the inactivation provided by peroxidase reducing substrates can be an explanation for the stimulative effect they exert on the cyclooxygenase reaction (118). On the other hand, the peroxidase reducing substrates should be generally strong cyclooxygenase inhibitors even at very low concentrations if the peroxidase and the cyclooxygenase share the same enzyme intermediate during the combined reaction (134-136). In particular this was expected for the excellent reducing substrates such as alkyaryl sulfides (114) but they did not inhibit the cyclooxygenase activity. This suggested that the relationship between the cyclooxygenase and peroxidase functions of PGH synthase is crucial for the understanding of the general mechanism, in particular for the understanding of the competency of the tyrosyl radical to activate and participate in the fatty acid oxygenation. The evaluation of the validity of the tyrosyl radical model and the quantitative relationship between the two activities was difficult to obtain for the reasons pointed out earlier in this chapter. The concept with the heme-dependent peroxidase reaction necessary to initiate the heme-independent cyclooxygenase reaction was tested in different ways in this study and experimental and theoretical evidence for alternative catalytic pathways are provided.

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CHAPTER TWO

STEADY-STATE KINETICS OF THE OXIDATION OF *P*-COUMARIC ACID BY PROSTAGLANDIN ENDOPEROXIDE SYNTHASE AND HYDROGEN PEROXIDE[*]

2.1. INTRODUCTION

p-Coumaric acid (CA)[**] is an important member of the hydroxycinnamate family which is widely distributed throughout the plant kingdom; and thus it is present in food and beverages derived from plants (1,2). Plant phenolases, laccases and peroxidases are enzymes responsible for the oxidative metabolism of hydroxycinnamate compounds (3,4). Among them, cell-wall-bound peroxidases are generally regarded as the natural catalysts of oxidative polymerization of hydroxylated cinnamyl alcohols (5,6).

CA is a natural antioxidant (7) as are eugenol, cinnamic acid and other natural aromatics (8). CA (9) and/or its synthetic analogs (10) are scavengers of the lipid peroxides and can protect against liver injury. Futhermore, CA is able to promote release of ferritin iron (11) and to activate NADH (6) and indoleacetic acid peroxidatic metabolism (12). CA isomers are the inhibitors of yeast phenylalanine ammonia-lyase (13), the activators of the electron transport and the inhibitors of photophosphorylation (14), and CA and its amides and esters are tyrosine kinase inhibitors and anticancer agents (15). Among other naturally occuring inhibitors of chemical carcinogenesis, *p*- and *o*-hydroxycinnamic acids cause mild inhibition of β-propiolactone induced neoplasia (16). Plant peroxidase, HRP, serves as a mediator in oxidative coupling of hydroxycinnamate reactive intermediates with anilines (17), polysaccharides (18,19) and amino acids and proteins (20,21).

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[**] Abbreviations: CA, *p*-coumaric acid; HRP, horseradish peroxidase; PGH synthase and PGHS, prostaglandin endoperoxide synthase (EC 1. 14. 99. 1); 20:4, arachidonic acid; PGG_2, prostaglandin G_2; PGH_2, prostaglandin H_2; DDC, diethyldithiocarbamate; ε, molar absorptivity; BSA, bovine serum albumin; 15-HPETE, 15-hydroperoxyeicosatetraenoic acid; TMPD, N,N,N',N'-tetramethyl-*p*-phenylenediamine; E, native enzyme; k_1, rate constant for Compound I formation; k_2, rate constant for one-electron reduction of Compound I; k_3, rate constant for one-electron reduction of Compound II; k_{-1}, rate constant for Compound I back to the native enzyme; k', rate constant for two-electron reduction of Compound I.

In this work it is shown that CA can be a reducing substrate for PGH synthase-peroxidase. We propose a ping-pong kinetic mechanism for the PGH synthase peroxidatic oxidation of CA that may be biologically important and we make a comparison of the PGH synthase and HRP systems in their reactions with CA. We describe steady-state experiments on a time scale where enzyme inactivation by hydrogen peroxide is not significantly affecting the reaction cycle.

2.2. MATERIALS AND METHODS

2.2.1. Materials

20:4, CA and DDC were purchased from Sigma. Tween 20 was purchased from J.T. Baker ; DE-53 ion exchange resin from Whatman. HRP (grade I) was obtained from Boehringer-Mannheim (A_{280nm}/A_{430nm} ratio of 3.1 after dialysis). The concentration of the HRP was determined at 403 nm using a molar absorptivity of 1.02×10^5 $M^{-1}cm^{-1}$ (22).

H_2O_2 (30%) was purchased from Fisher. The concentrations of H_2O_2 diluted stock solutions were determined by the peroxidase assay (23) and / or by absorbance change at 240 nm using an ε of 43.6 $M^{-1}cm^{-1}$ (24).

2.2.2. Isolation of PGH Synthase from Ram Seminal Vesicles

PGH synthase was isolated and purified using published procedure (25, 26). Macerated ram seminal vesicles (100 g) were rinsed 2-3 times with 20 ml 1.15% KCl, suspended in 0.25 M sucrose in 0.1 M phosphate buffer, pH 7.4 (300 ml), and homogenized in a Waring blender at maximum speed for 1 min. The blender was washed out with 50 ml sucrose solution and the mixture was homogenized 3 min with a IKA-Werk, Ultra Turrac T25 homogenizer at medium speed. The homogenate was then centrifugated at 11 500 r.p.m. in a Beckman T2-21 centrifuge at 4°C for 20 min. The supernatant was filtered through three layers of cheesecloth two times. The filtrate was centrifuged at 35 500 r.p.m. in a Beckman 45 Ti rotor with Beckman L8-70M ultracentrifuge for 90 min at 4°C. The pellet was scraped into a solubilizing buffer of 180 ml of 5 mM Tris-HCl, pH 8.0, 1% Tween-20 and 100 µM DDC. The mixture was stirred for 1 hour and centrifugated for 90 min at 35 500 r.p.m. The supernatant was collected and concentrated to 15-25 ml using an Amicon ultrafiltration apparatus and an XM-50 Diaflo membrane. The crude enzyme concentrate was then mixed with DE-53 ion exchange gel preequilibrated with 5 mM Tris-HCl buffer, pH 8.0, 0.1% Tween-20, and 100 µM DDC (buffer A). The mixture was stirred for 1 hour under vacuum and the protein-loaded gel was put into 30 x 1.4 cm column. The column was washed with 150 ml buffer A at flow rate 1.6 ml/min. The elution of PGH synthase was started by applying a linear ionic strenght gradient from 5 mM to 100 mM Tris-HCl. 250 ml of buffer A was starting buffer and 250 ml of buffer B (100 mM Tris-HCl, pH 8.0, 0.1 % Tween-20 and 100 µM DDC) was the finishing buffer. 16 ml fractions were collected and the protein content and specific cyclooxygenase activity were determined. The protein concentration was determined by the BioRad protein assay using BSA as a standard. The concentration of heme was determined at 410 nm using a molar absorptivity of

123 $mM^{-1}cm^{-1}$ (27). The specific cyclooxygenase activity was determined by Yellow Spring Instrument Model 53 Oxygen Monitor equipped with a Clark-type polarographic electrode. The cyclooxygenase reaction was initiated by adding 200 μM 20:4 to 250 μM enzyme, 1 μM hematin and 1 mM phenol in 0.1 M phosphate buffer, pH 8.0. The reaction rate was determined from the maximum slope of the oxygen consumption curve by assuming that 2 mol of oxygen were consumed per mole of 20:4. The oxygen concentration in 0.1 M phosphate buffer was 0.23 mM at 30°C (28).The fractions with specific activity higher than 25 μM 20:4/(mg protein-min) were collected and concentrated by ultrafiltration on an XM-50 membrane and stored at -70°C. The enzyme used in these kinetic experiments was a 82% holoenzyme having a specific cyclooxygenase activity of 69 μM O_2/(mg protein–min). No hematin was added.

2.2.3. Effect of CA on the PGH Synthase Cyclooxygenase Reaction

The effect of CA on the PGH synthase cyclooxygenase activity was determined by monitoring oxygen uptake during the reaction of 200 μM 20:4, with either 17, 18, or 20 nM enzyme and CA in various concentrations in 0.1 M phosphate buffer pH 8.0 and without hematin. The initial reaction rates in the presence and absence of CA were compared and plotted as a function of [CA].

2.2.4. PGH Synthase and HRP Peroxidase Reactions

Optical absorption measurements on a conventional time scale were made on a Cary 219 spectrophotometer. To determine if CA is a reducing substrate of PGH synthase peroxidase, separate assays with PGH synthase/H_2O_2 and HRP/H_2O_2 systems were performed by comparing spectral changes in the 227-387 nm region. The drop in CA absorbance at 286 nm was used as a parameter in setting optimal conditions for the kinetic steady-state experiments. There were no significant spectral interferences from H_2O_2 (up to 8 mM) and PGH synthase (up to 0.1 mM) with the CA spectra. However, there was some overlap in the far UV region at the lowest CA concentration used (5 μM) so that the absorbance at 300 nm was chosen as more convenient for the kinetic steady-state measurements.

2.2.5. Determination of Molar Absorptivity of CA

The molar absorptivity of CA at 300 nm was determined at 25±1°C in 0.1 M phosphate buffer (pH 8.0). The CA was recrystallized from hot water and had a purity of 98% as determined by elemental microanalysis. The molar absorptivity of CA calculated from four measurements was 17.2 ± 0.9 $mM^{-1}cm^{-1}$.

2.2.6. Steady-State Kinetic Measurements

The steady-state experiments were performed on a Photal (formerly Union Giken) RA601 stopped flow apparatus. One compartment was used for the H_2O_2 solutions, the other for the PGH synthase and CA all in 0.1 M phosphate buffer (pH 8.0). After 3 min of incubation at 25±0.5°C, the initial rate of CA disappearance was determined at 300 nm from the slope of the linear trace obtained after fast (2 ms) mixing of all the components. The initial rates were determined up to 1.5 s after the reaction was started. A stoichiometry of 2 mol CA oxidized per mol of H_2O_2 reduced was used in calculations.

2.2.7. Curve Fittings and Calculations

A non-linear regression data analysis program (ENZFITTER, Elsevier-Biosoft, Cambridge, UK) was used for curve fittings and calculations. The initial rate (v) data versus substrate concentration (S) were fitted an equation of the form

$$\frac{v}{[E]_{tot}} = \frac{A\,[S]}{B + [S]} \qquad [2.1]$$

to determine the values of the parameters A and B.

In the PGH synthase cyclooxygenase reaction, the variables v and [S] represent the rate of oxygen consumption and [CA] respectively. Parameter A is k_{cat} for cyclooxygenase activity; parameter B is A_{50}, i.e., the value of [CA] that produces a half of the maximum rate of the oxygen consumption.

In the PGH synthase peroxidase reaction, the variables v and [S] are the rate of H_2O_2 disappearance and $[H_2O_2]$ respectively. Parameters A and B were fitted to the linear equations as functions of [CA].

2.3. RESULTS

2.3.1. Stimulation of PGH Synthase Cyclooxygenase Activity by CA

CA stimulated the cyclooxygenase activity of PGH synthase as determined by measuring the oxygen consumption during the catalytic oxidation of 20:4. The stimulation of cyclooxygenase activity was dependent upon CA concentration and showed saturation behaviour (Figure 2.1). The k_{cat} for the oxygen uptake in the presence of CA was $87 \pm 3\ s^{-1}$ when 200 μM 20:4 was used. The concentration of CA necessary to produce 50% stimulation (A_{50}) was 350 ± 30 μM. Concentrations of CA up to 1.8 mM did not inhibit the oxygen uptake. Measurements above 1.8 mM CA were limited by the low solubility of CA in phosphate buffer.

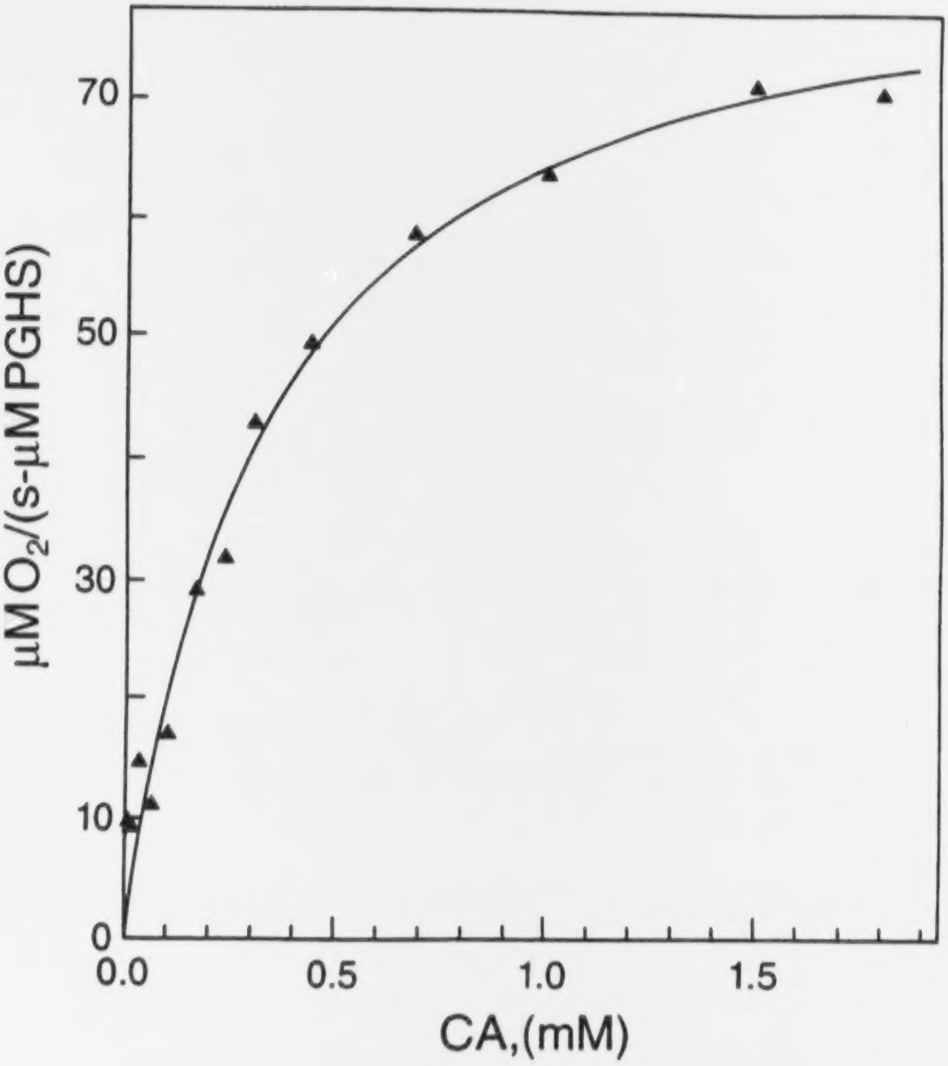


Figure 2.1. Plot of μM O_2 consumed per second per μM PGH synthase *versus* [CA] at 30 ± 1°C in 0.1 M phosphate buffer (pH 8.0) with 200 μM 20:4 and without hematin.

2.3.2. CA oxidation by HRP and PGH synthase

CA stongly absorbs in the UV region with a maximum at 286 nm and a shoulder at 294 nm at pH 8.0 (Figure 2.2, A-D, a). CA is not oxidized spontaneously with H_2O_2 but in the presence of catalytic amounts of either HRP or PGH synthase it is oxidized very quickly, giving identical products (Figure 2.2, A-b and D-b). The isosbestic points between CA and its product are at 254 and 324 nm in the HRP/H_2O_2 system and at 253 and 323 nm in the PGH synthase/H_2O_2 system, essentially the same within experimental error. The product has a broad maximum at 290 nm and a shoulder at 320 nm, and does not react further under the given experimental conditions. The oxidation of CA was complete at low concentration of H_2O_2 and high concentration of PGH synthase (Figure 2.2, C and D) but it was inhibited at high concentration of H_2O_2 and low concentration of PGH synthase (Figure 2,B).

2.3.3. Initial Rate Measurements for the Reaction of CA with PGH Synthase and H_2O_2

Preliminary tests were made to find the relationship between rate, total enzyme concentration and concentrations of both substrates. The rate of CA disappearance is a linear function of the enzyme concentration in the range of 20 to 50 nM enzyme at fixed concentrations of CA and H_2O_2. The intercept is not significantly different from zero and the slope is 41±1 µM CA (s·nM PGH synthase)$^{-1}$. At concentrations above 50 nM a saturation in PGH synthase peroxidase activity was observed. A linear dependence of the initial rates on CA concentration at fixed PGH synthase and H_2O_2 concentrations is also obtained with zero intercept and a slope of 2.1±0.1 µM PGH synthase•s^{-1}. A range in reducing substrate concentration of at least one order of magnitude was covered.

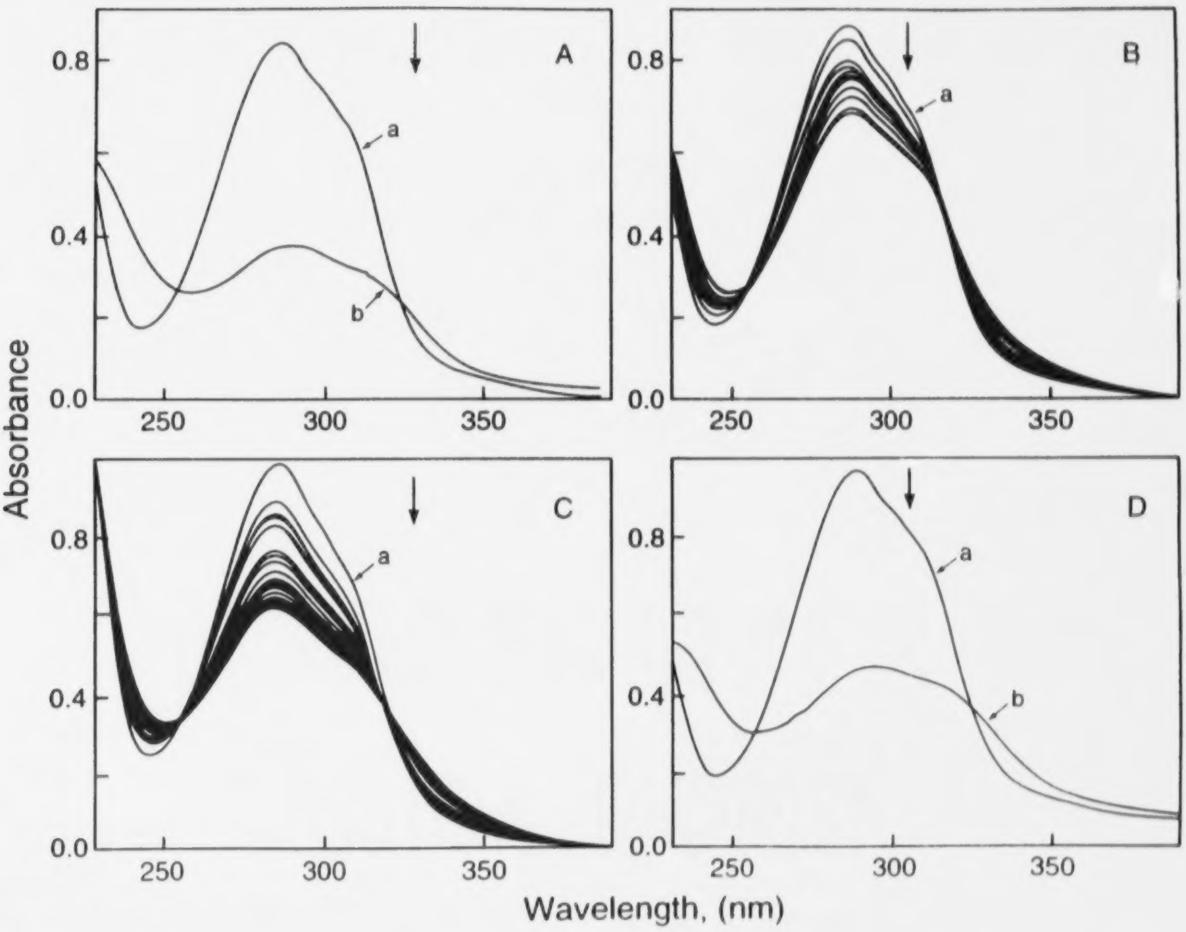


Figure 2. 2. The oxidation of CA by HRP and PGH synthase. **A.** Initial (a) and final (b) spectra of CA (50 μM) after its reaction with 1 mM H_2O_2 and 25 nM HRP ; **B.** Repetitive scans (5 nm/s) of CA (50 μM) during its reaction with 1 mM H_2O_2 and 25 nM PGH synthase; **C.** Repetitive scans (5 nm/s) of CA (50 μM) during its reaction with 0.25 mM H_2O_2 and 50 nM PGH synthase and **D.** spectra of CA under conditions as in **C**.

The assay period was short enough (1.5 s) to ensure that only a small fraction of the CA was utilized. Over the utilized concentration range of CA, 3-8% of the CA was reacted after 1.5 s. This was ascertained from the initial drop in absorbance at 286 nm by using rapid scan measurements (data not shown).

The initial rate of CA disappearance was measured at 300 nm immediately after the steady-state conditions had been established: the concentration of the enzyme intermediate - PGH synthase-compound II - rapidly reaches its maximum value which then remains constant for a time interval over which an initial steady-state rate of substrate disappearance can be measured. The change in absorbance was monitored at 426 nm (the isosbestic point between PGH synthase-compound I and PGH synthase-native enzyme) and 419 nm (the absorption maxima of PGH synthase-compound II) (29). The scan of the absorbance at 426 nm *versus* time (Figure 2.3) clearly indicates that PGH synthase-compound II is the dominant enzyme intermediate under steady-state conditions.

Initial rate data can be represented by an empirical linear equation of the form

$$S = \alpha + \beta t \qquad [2.2]$$

S represents the concentration of the CA that has disappeared at time t. The coefficient α is the intercept; if negative, α could represent a lag period but if positive it could represent contamination by the product. As can be seen in Figure 2.4, both situations existed but they did not significantly affect the initial rate determination from slopes β over the 0.5-1.5 s time scale. Applying Beer's law, the initial rate of CA disappearance has been calculated from the change in absorbance as a function of time. Average values of β were determined from at least 5 measurements in which the relative error was usually less than 8%.

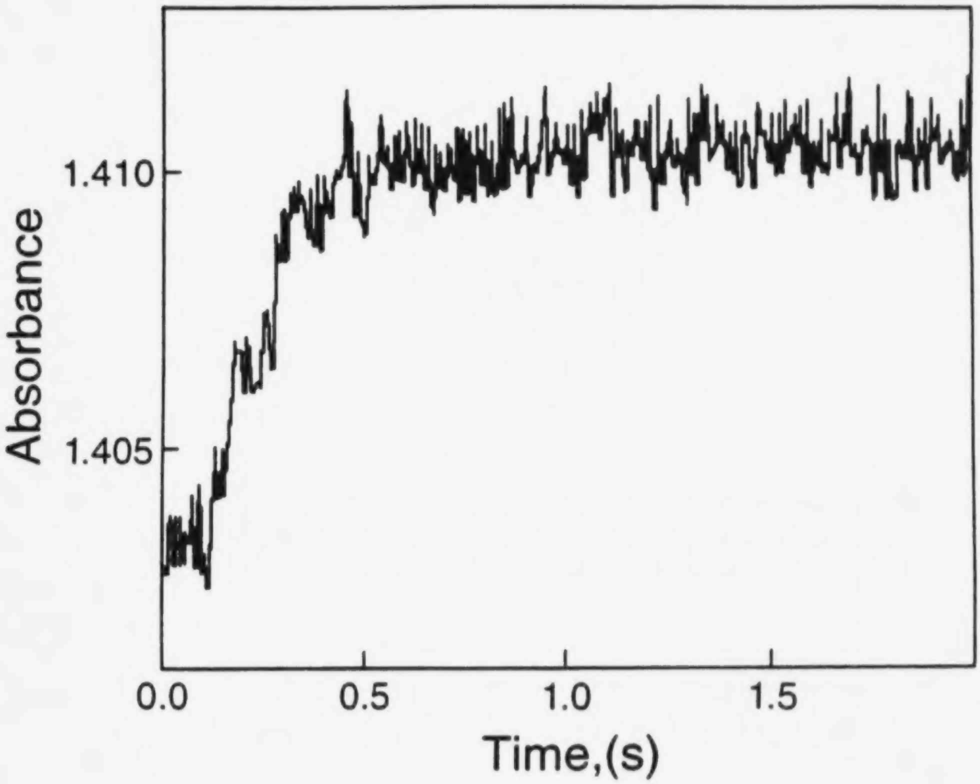


Figure 2. 3. Kinetic trace of PGH synthase (0.3 μM) absorbance at 426 nm in the presence of 30 μM CA and 0.5 mM H_2O_2 in 0.1 M phosphate buffer (pH 8.0) at 25 ± 0.5°C

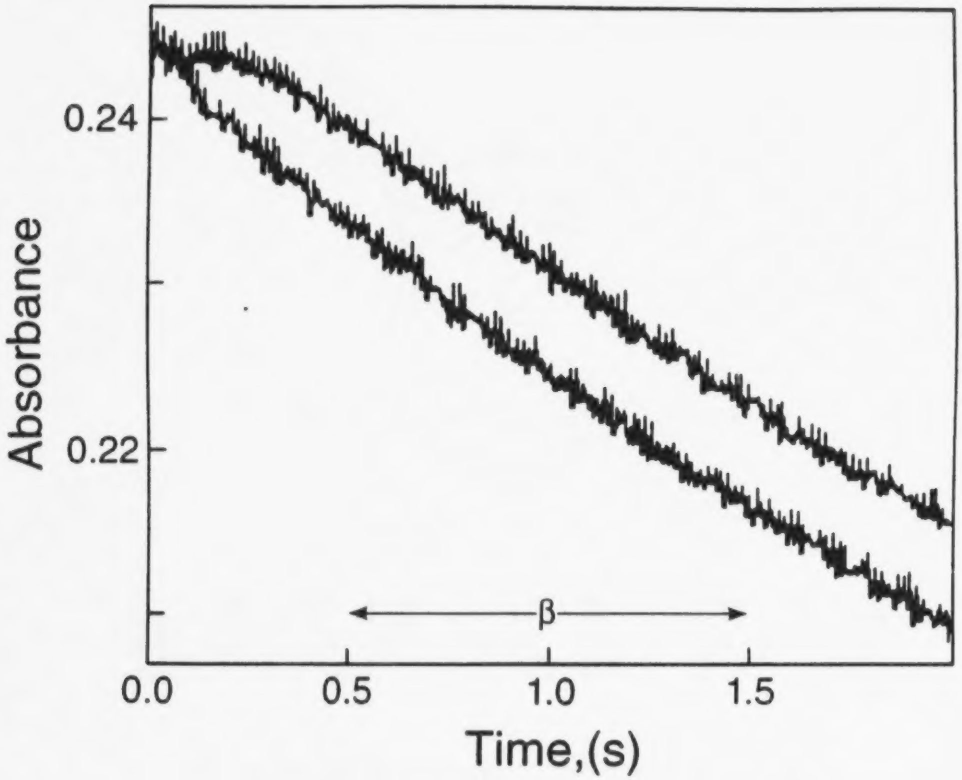


Figure 2. 4. Repetitive kinetic traces of CA absorbance at 300 nm during its oxidation with PGH synthase and H_2O_2. The reaction mixture contained 20.35 μM CA, 0.382 mM H_2O_2 and 25 nM PGH synthase in 0.1 M phosphate buffer (pH 8.0) at 25 ± 0.5°C; β is the coefficient from eq 2.2

When the reaction period was extended to 5 s a departure from linearity was observed (Figure 2.5). By increasing the H_2O_2 concentration the rate of the reaction was increased and the departure from linearity was seen in even shorter times. The points of departure are labelled A-D in Figure 2.5.

2.3.4. Steady-State Kinetics for the Reaction of CA with PGH Synthase and H_2O_2

The linear portions in the traces on a short time scale (Figure 2.4) allowed us to obtain the kinetic data presented in Figure 2.6. In the range of 5.4 to 55.2 μM CA the initial rate was dependent upon both H_2O_2 and CA concentrations and showed saturation behavior. After fitting the experimental data to eq. 2.1, parameters A and B were obtained (Table 2.1). Kinetic parameters obtained from steady-state analysis can be fitted to a rectangular hyperbola (eq. 2.1) by using the operational equations from a) the irreversible reaction mechanism which is widely accepted for HRP (30) or b) in the manner of reversible Michaelis-Menten reaction kinetics (24,31) that is incorrectly applied for HRP systems (6,32).

Based on the results obtained in this study the most plausible mechanism for the oxidation of CA by H_2O_2 catalyzed by PGH synthase is the irreversible reaction mechanism (Mechanism I) presented in Figure 2.7. The distinction between the mechanisms is based upon the ratio A/B. In Mechanism I, the irreversible mechanism, this ratio is equal to k_1 (Appendix A, eq A.7) and is independent of [CA]. For both Mechanisms II and III (Figure 2.7, and Appendix eqs A.15 and A.21) the ratio of A/B is a hyperbolic function of [CA] which is contrary to the experimental results. A plot of A versus B (Table 2.1, Figure 2.8) is linear with a slope of $k_1 = (4.6 \pm 0.1) \times 10^5\ M^{-1}s^{-1}$ and an intercept not significantly different from zero.

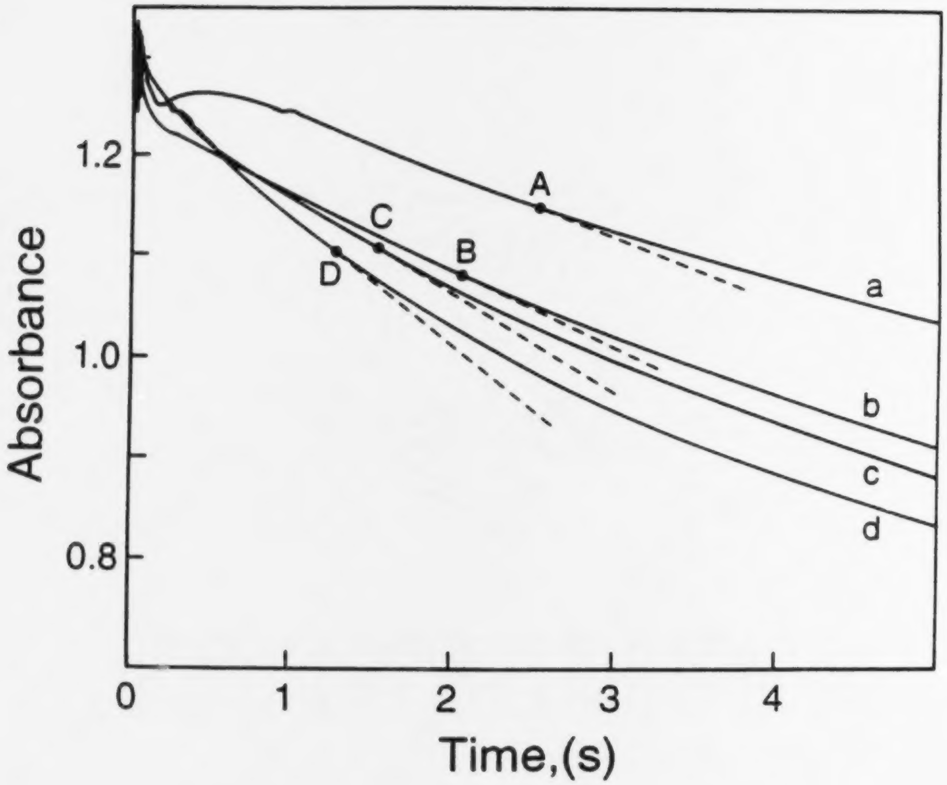


Figure 2. 5. Hydrogen peroxide dependent oxidation of CA. The reaction mixture contained 69.85 μM CA, 51 nM PGH synthase and a) 0.271 b) 0.596 c) 1.953 and d) 3.255 mM H_2O_2 in 0.1 M phosphate buffer (pH 8.0) at 25 ± 0.5°C. Kinetic traces obtained at 300 nm. A-D: the points of departure from linearity.

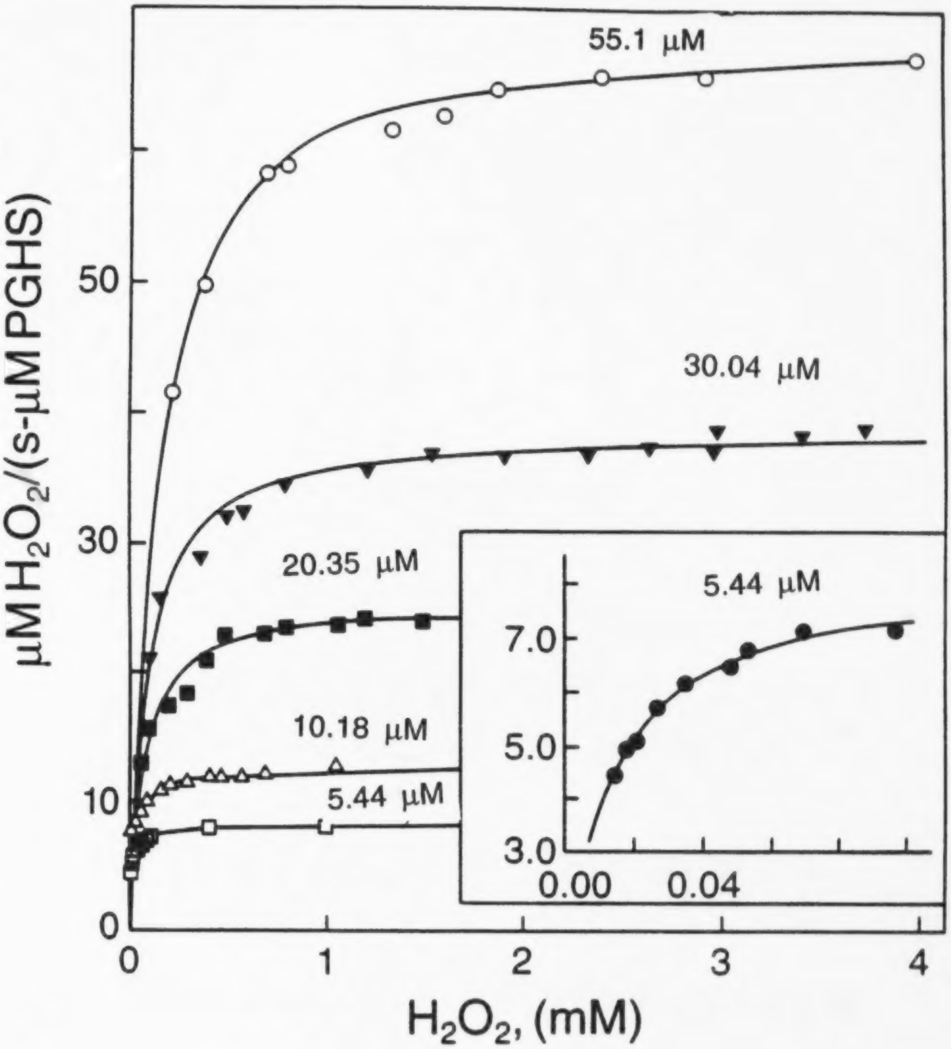


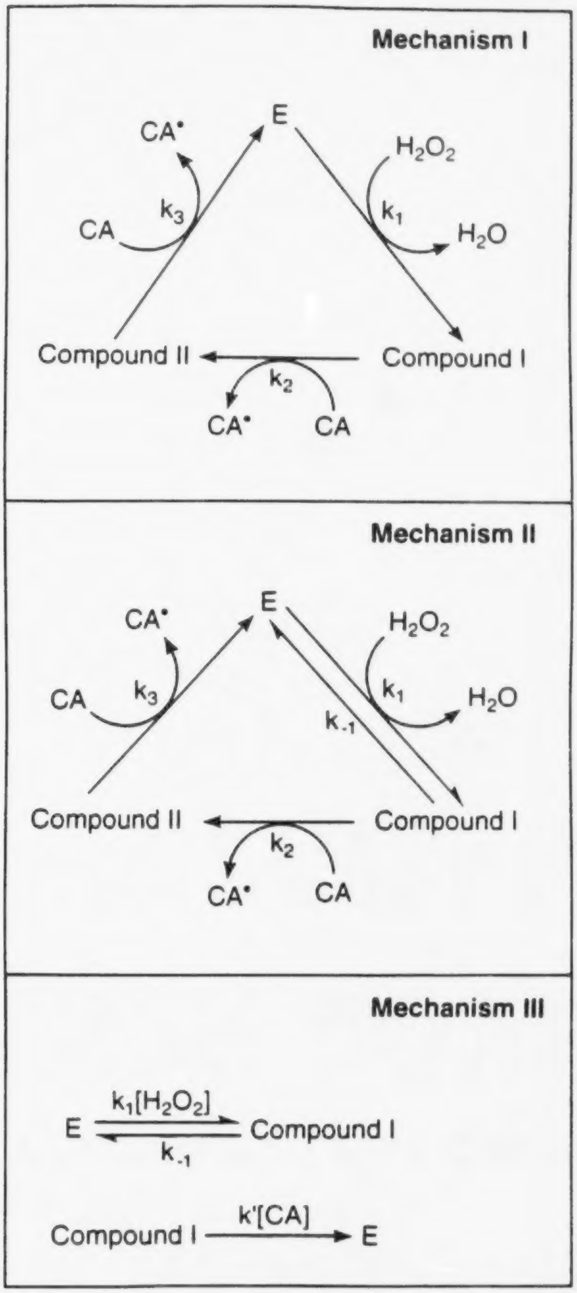
Figure 2. 6. Steady-state initial rates at unit [PGH synthase]$_o$ of the reduction of the H_2O_2 obtained at five concentrations of CA. *Inset:* The initial part of the curve corresponding to 5.44 μM CA. The axes are the same as in the main part of the Figure.

Table 2. 1. Steady-State Parameters for the Peroxidase Reaction of PGH synthase with H_2O_2 and CA Obtained from Plotting The Initial Rate of H_2O_2 Disappearance against H_2O_2 Concentrations at Constant Concentrations of CA [a]

CA (μM)	A parameter [b] (s^{-1})	B parameter (μM)
5.44	8.1 (0.1) [c]	11.9 (0.1)
10.18	12.57 (0.02)	23.5 (0.1)
20.35	25.3 (0.2)	60.6 (0.1)
30.04	38.7 (0.3)	85.6 (0.1)
55.19	70.6 (0.5)	148.0 (0.1)

[a] All measurements were performed in 0.1 M phosphate buffer (pH 8.0) at 25 ± 0.5°C.

[b] Parameters A and B were obtained after fitting the eq. 2.1. to the experimental data. [c] Standard deviation.


Mechanism I
E
H_2O_2
k_1
H_2O
$CA^•$
k_3
CA
Compound II
Compound I
k_2
$CA^•$
CA
Mechanism II
E
H_2O_2
k_1
k_{-1}
H_2O
$CA^•$
k_3
CA
Compound II
Compound I
k_2
$CA^•$
CA
Mechanism III

$$E \underset{k_{-1}}{\overset{k_1[H_2O_2]}{\rightleftharpoons}} \text{Compound I}$$

$$\text{Compound I} \xrightarrow{k'[CA]} E$$

Figure 2. 7. The three possible mechanisms for the oxidation of CA by H_2O_2, catalyzed by PGH synthase.

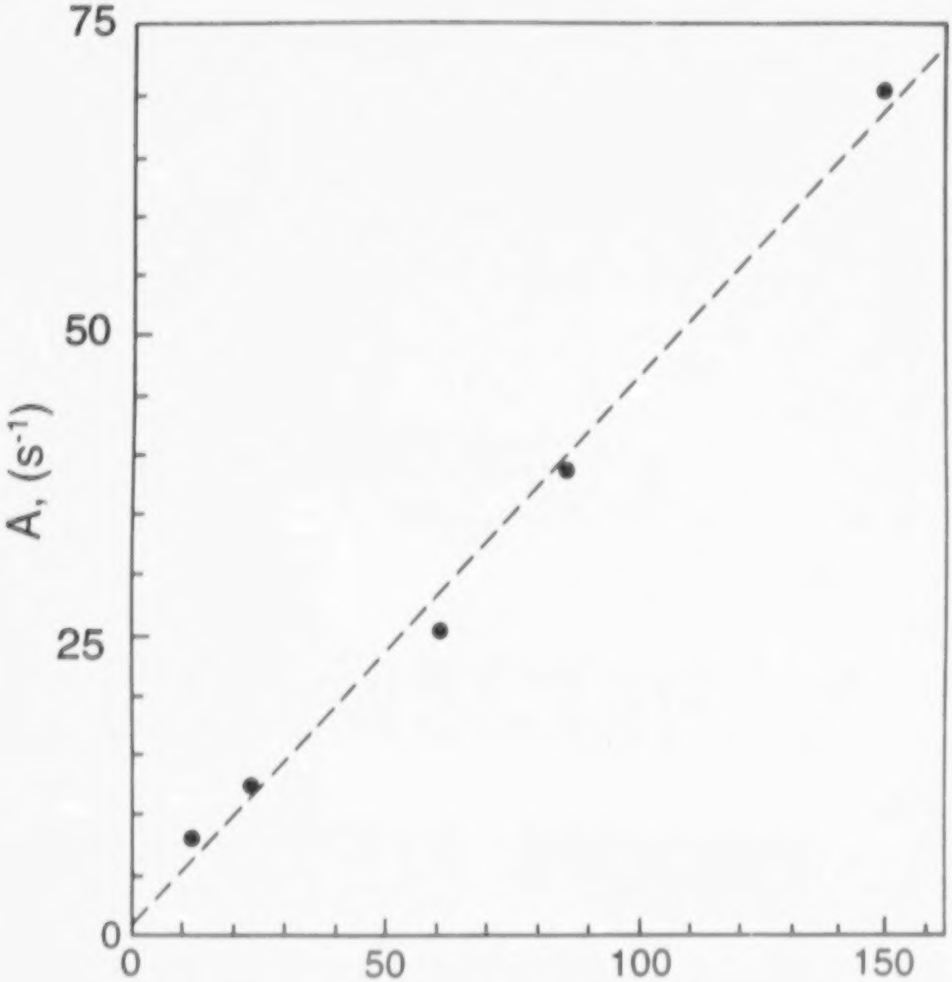


Figure 2.8. The linear dependence of A parameter versus B parameter for peroxidase activity, showing that the mechanism I is correct. The numerical values are from Table 2.1. The slope of the line is $(4.6 \pm 0.1) \times 10^5\ M^{-1}s^{-1}$.

Recovery of the native PGH synthase from PGH synthase-compound II is the rate controlling step. Consequently $k_2 \gg k_3$ and a simplified rate eq 2.3 is obtained:

$$\frac{\frac{-d[H_2O_2]}{dt}}{[E]_{tot}} = \frac{k_3\ [CA]\ [H_2O_2]}{\frac{k_3}{k_1}\ [CA] + [H_2O_2]} \qquad [2.3]$$

The parameters A and B become the following :

$$A = k_3\,[CA] \qquad [2.4]$$

$$B = \frac{k_3}{k_1}\ [CA] \qquad [2.5]$$

Plots of parameter A or B as a function of [CA] thus yielded straight lines with slopes of k_3 ($1.27 \pm 0.04\ \mu M^{-1}s^{-1}$) and k_3/k_1 (2.80 ± 0.08) respectively. As expected from the forms of eqs 2.4 and 2.5, the intercepts were not significantly different from zero. The rate constants $k_1 = (4.6 \pm 1\ )x10^5\ M^{-1}s^{-1}$ and $k_3 = (1.27 \pm 0.1)x10^6\ M^{-1}s^{-1}$ then were obtained. The values of k_1 determined directly from Mechanism I (Appendix A, eq A.7) or by using the simplified version (eqs 2.4 and 2.5) are in a good agreement.

2.4. DISCUSSION

Our kinetic and spectroscopic data demonstrate that the naturally occuring hydroxycinnamate derivative, CA, functions as a reducing substrate of PGH synthase peroxidase and stimulates PGH synthase cyclooxygenase activity.

On longer time scales the inactivation of PGH synthase, particularly in the presence of higher concentrations of hydroperoxides, has a significant effect on a normal catalytic cycle so that product yields were either underestimated (33,34) or kinetic parameters were corrected for the inactivation (24). On the other hand, rapid scan spectral and transient-state kinetic studies (29,35-37) showed that reducing substrates are capable of protecting the enzyme from oxidative inactivation. This prompted us to believe that the difficulties that usually appear in steady-state kinetic studies due to rapid PGH synthase inactivation can be overcome by using stopped-flow spectrophotometry. In the PGH synthase peroxidase reaction with H_2O_2 as an oxidizing substrate and CA as a reducing substrate we were able to obtain accurate kinetic data without inhibition on a time scale up to 1.5 s after fast mixing all of the components. H_2O_2 was chosen as an oxidizing substrate instead of organic hydroperoxides because it does not inactivate PGH synthase as much as the organic hydroperoxide 15-HPETE. The inactivation constants are 30 $M^{-1}s^{-1}$ for H_2O_2 versus 2×10^4 $M^{-1}s^{-1}$ for 15-HPETE (31).

We propose the irreversible reaction mechanism for the PGH synthase peroxidase reaction (Figure 2.7, Mechanism I) for the first time for steady state conditions. The results obtained earlier in our laboratory under transient-state conditions (29,35-39) were all interpreted in terms of Mechanism I.

Mechanism I is a three-step cycle involving a two-electron enzyme oxidation (native enzyme → compound I) followed by two one-electron reductions (compound I → compound II followed by compound II → native enzyme). Our rate constant determined for the first step (in detail, native enzyme + H_2O_2 → compound I + H_2O) is $(4.6 \pm 0.1) \times 10^5$ $M^{-1}s^{-1}$. This is 1.5 orders of magnitude higher than what has been estimated from (40) a kinetic simulation of the reaction mechanism with tyrosyl radical formation as one of the steps in the PGH synthase cycle (1×10^4 $M^{-1}s^{-1}$ without a reducing substrate) . It is 0.5 order of magnitude greater than the value of 9.1×10^4 $M^{-1}s^{-1}$ obtained in a study using H_2O_2 and TMPD as the oxidizing and reducing substrates (24). In the latter study the normal peroxidase cycle with three irreversible steps was approximated by Mechanism III containing one reversible and one irreversible step. An apparent Michaelis constant K_M^{app} was introduced and the reaction was classified as obeying an ordered mechanism. Peroxidase kinetics obey a modified ping pong mechanism and Michaelis constants, in the strict sense, are meaningless for irreversible reactions (41). The different results obtained by Kulmacz (24) can partly be attributed to the different mechanisms and partly to the different time scales; inactivation was a factor on the longer time scale.

Our results also show that the oxidation product of CA is identical with the product obtained in the HRP/H_2O_2 system, for which the irreversible reaction mechanism involving two enzyme intermediates is well established (42). Our rate constant for the reaction of PGH synthase-compound II, k_3, with CA ($(1.27 \pm 0.1) \times 10^6\ M^{-1}s^{-1}$) is comparable with the rate constant for the reaction HRP-compound II with CA ($5 \times 10^6\ M^{-1}s^{-1}$) (43).

It is also clear that the inactivation of PGH synthase is irrelevant on a time scale up to 1.5 s and up to 5 mM H_2O_2. Under these conditions CA is able to protect the enzyme from oxidative damage at concentrations as low as 5 μM (Figure 2.6). A recent investigation showed that phenol can completely prevent doublet and partially prevent formation of the singlet tyrosyl radical signal without inhibiting cyclooxygenase activity (44). It also has been suggested (36) that the tyrosyl radical is not a significant intermediate when any of the reducing substrates phenol, hydroquinone or DDC are present. Our results are consistent with these findings and with the view that hydroperoxide-dependent generation of the tyrosyl radical represents an inactivation step rather than the initiation step in the cyclooxygenase reaction.

The ability of CA to serve as a reducing substrate for PGH synthase peroxidase correlates well with its ability to stimulate PGH synthase cyclooxygenase activity. CA prevents autoinactivation of the PGH synthase probably not only by minimizing the level of unproductive higher oxidation states of PGH synthase but also by reducing the peroxyl radical of PGG_2, as it has been proposed for acetaminophen (45) and substituted phenols (35). Also it is known that CA can scavenge lipid hydroperoxides (9,10). The rate constant of the overall reaction from 20:4 to the formation of PGG_2 was estimated to be 4×10^4 $M^{-1}s^{-1}$ at 25°C (37) which is two orders of magnitude slower than the conversion of PGH synthase-compound II by CA. If carbon-centered radical formation (46) is the rate controlling step in the cyclooxygenase reaction then 20:4 is unable to compete with CA for the enzyme intermediate compound I. It is reasonable to expect that CA reacts with PGH synthase-compound I in a manner similar to HRP-compound I, for which the rate constant is 1.2×10^7 $M^{-1}s^{-1}$ (43).

Yet another aspect of the observed stimulation of 20:4 metabolism by CA needs to be considered. Hsuanyu and Dunford (37) established a quantitative relationship between both stimulatory and inhibitory abilities of phenolic compounds for the PGH synthase cyclooxygenase with electronic and hydrophobic properties of phenolic ring substituents. The action of phenols is a complex combination of structural and electronic effects resulting in a competition of phenols with the 20:4 not only for the enzyme higher oxidation states but also for the 20:4 hydrophobic binding site. The hydrophobic effect strongly inhibits PGH synthase cyclooxygenase activity. Consequently, compounds such as *p*-hydroxybenzoic acid did not exibit inhibition partly due to the presence of a very strong hydrophilic carboxyl group. In the case of CA the stimulatory effect was also observed (Figure 2.1). At pH 8, the carboxyl group of CA is deprotonated and the hydroxyl group partially deprotonated (pK_1 and pK_2 are 4.64 and 9.45 respectively) (47) which increases the hydrophilicity of CA and diminishes its competition for the 20:4 binding site.

This study shows that PGH synthase can play a role in biologically important events such as oxidation of naturally occurring CA and related compounds. These observations are consistent with one-electron oxidations by PGH synthase-compound I and II to form phenoxyl radicals similarly to other phenols (37). The clear isosbestic point obtained in CA UV spectrum (Figure 2.2, B) indicates that a single product is formed during its reaction with PGH synthase and H_2O_2. At least three ways of dimerization of the CA radicals are possible:

a) through an *o,o'* linkage, as in case of eugenol (48), ferulic acid (19) , phenol (49) and tyrosine (50) oxidation with plant peroxidases;

b) through a β,β' linkage as in the case of ferulic and caffeic acid oxidation with a peroxidase specific for this reaction from *Bulpeurum salicifolium* (51) and

c) through β-O-4 linkage where similar products were formed when methylpherulate and isoeugenol were dimerized in the HRP/H_2O_2 system (48).

Unlike ferulic acid oxidation with HRP/H_2O_2, in a PGH synthase catalyzed reaction the ferulic acid undergoes O-demethylation to give caffeic acid similarly to the cytochrome P-450 catalyzed reaction (48). Eugenol inhibits PGH synthase activity (52) and chemiluminescence (53). During the HRP catalyzed oxidation of eugenol a transient quinone metide metabolite is formed (54). CA enhances luminiscence in the HRP/luminol/H_2O_2 system (43). Our data showed that CA stimulates PGH synthase activity and it seems that cytotoxic quinone metide (a yellow colored intermediate with absorption maxima at 346 nm) is not the reaction intermediate in peroxidative oxidation of the CA by PGH synthase and HRP.

In conclusion, some important points concerning the mechanism of PGH synthase peroxidase have been established by using the stopped-flow method for steady-state kinetic measurements. An irreversible reaction mechanism similar to that for HRP is proposed. With CA and H_2O_2 as co-substrates, we were able to obtain an accurate turnover number and rate constant for the rate controlling step which is the conversion of PGH synthase-compound II to the native enzyme and for the PGH synthase-compound I formation. In view of the limitations imposed by the steady-state method it is not possible to draw any conclusions about the relative magnitude of the rate of PGH synthase-compound I conversion to the PGH synthase-compound II. However, the overall peroxidatic activity of PGH synthase for a substrate such as CA was defined and compared to the HRP system. In the case of CA the reaction product is the same as that obtained in the HRP catalyzed reaction and the reaction rates are comparable. Further studies are needed to yield more details about the PGH synthase peroxidase reaction mechanism. Even though PGH synthase and HRP can utilize the same reducing substrate, the products and intermediates can be dramatically different as in case of ferulic acid (48,51) or eugenol (48,54).

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CHAPTER THREE

BINDING AND OXIDATION KINETICS OF CAFFEIC ACID BY PROSTAGLANDIN ENDOPEROXIDE SYNTHASE. POTENTIAL ROLE IN REGULATION OF PROSTAGLANDIN BIOSYNTHESIS*

3.1 INTRODUCTION

A model for the prostaglandin endoperoxide synthase (PGH synthase)** active site has been developed that includes: (a) separate cyclooxygenase (arachidonic acid and nonsteroidal antiinflammatory drugs) and peroxidase (peroxide) binding sites; (b) one heme coordinated by one histidine, and (c) a tyrosine residue as initiator of cyclooxygenase activity (1). The suggested model of the PGH synthase active site does not show how the enzyme accommodates the peroxidase reducing substrates. The site of binding of the reducing substrate and how it affects both the cyclooxygenase and peroxidase functions is still unknown. A combined transient state kinetic study for the peroxidase function, using a series of monophenols, and a steady state kinetic study for the oxygenase function of PGH synthase showed that the hydrophobic properties of the reducing substrates significantly modulate cyclooxygenase activity but not peroxidase activity (2). Our results presented here show that the hydrophobic properties of the reducing substrate can also affect the peroxidase function of PGH synthase.

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**Abbreviations: PGH synthase and PGHS, prostaglandin endoperoxide synthase (EC 1. 14. 99. 1); CAF, caffeic acid; ε_{CAF} and $\varepsilon_{CAF,pr}$ molar absorpitivities of caffeic acid and reaction product of caffeic acid; $v/[E]_{tot}$, velocity per unit total enzyme concentration; PGHS-I, prostaglandin endoperoxide synthase-compound I; PGHS-II, prostaglandin endoperoxide synthase-compound II; k_{cat} is the maximum turnover number for the cyclooxygenase reaction; $k_{cat}^{app}$and K_m^{app} are constants for the peroxidase reaction of caffeic acid.

We have selected a catechol derivative, caffeic acid, for detailed kinetic study. Caffeic acid is a chain-breaking antioxidant (3) and an efficient reductant of intracellular iron (4). It inhibits the release of reactive oxygen species by human neutrophils (5), prevents tumor promotion (6), and produces a sustained inhibition of peroxidative oxidation of indole-3-acetic acid (7). Depending on the conditions, caffeic acid inhibits or enhances hydroxyl radical formation from the Fenton reaction (8, 9), and inhibits leukotriene biosynthesis (10, 11). As a catechol derivative, caffeic acid can model catecholamine oxidation during prostaglandin biosynthesis, for which the mechanisms and the kinetic data are still unavailable (12, 13).

A further rationale for studying the reaction of caffeic acid was to test its chelating ability toward ferric ion to determine whether it would coordinate the active site iron and form dead-end complexes. We also wanted to confirm the previous findings (14) that caffeic acid is a stable product formed from ferulic acid in a cytochrome P-450 type of action of PGH synthase. Comparing the product spectra obtained with PGH synthase with the product spectra obtained with horseradish peroxidase, we found instead that caffeic acid is not the product of ferulic acid oxidation. Ferulic acid oxidation is peroxidatic in nature, not a cytochrome P-450 type of reaction. Caffeic acid is both a competent electron donor to the higher oxidation states of enzyme and a competitive inhibitor of the hydrogen peroxide reaction with PGH synthase.

3.2. MATERIALS AND METHODS

PGH synthase was prepared by reported methodology (Chapter 2, 15, 16). An 89 % holoenzyme having a specific activity of 76 μM O_2(mg protein min)$^{-1}$ was used in kinetic experiments. The concentration of PGH synthase was determined by measuring absorbance at 410 nm where ε_{PGHS} = 123 mM^{-1}cm^{-1} (17), and protein content by the BioRad protein assay using bovine serum albumin as a standard.

Horseradish peroxidase was purchased from Boehringer-Mannheim (A_{280nm} / A_{430nm} = 3.1). A molar absorptivity of 1.02×10^5 M^{-1} cm^{-1} at 403 nm (18) was used for spectrophotometric determination of enzyme concentration.

Arachidonic, ferulic and caffeic acids were obtained from Sigma, and hydrogen peroxide from Fisher. Ferulic and caffeic acids were recrystallized from water. The concentration of caffeic acid was determined at 310 nm using ε_{CAF} = 12.47 ± 0.01 mM^{-1}cm^{-1} from our own measurements. The molar extinction coefficient ε_{CAF} was determined over the range 5-200 μM caffeic acid in 0.1 M phosphate buffer, pH 8.0, at room temperature. The concentration of H_2O_2 was also determined spectrophotometrically using $\varepsilon_{H_2O_2}$ = 43.6 M^{-1}cm^{-1} at 240 nm (19).

Cyclooxygenase activity was assayed by using a Yellow Springs Instrument Co. model 53 oxygen monitor. The effect of ferulic and caffeic acids on the cyclooxygenase function of PGH synthase was followed by using 20 nM enzyme, 0.2 mM arachidonic acid, and either ferulic or caffeic acids in varying concentrations. The assay mixture was thermostated at 30 ^{o}C and the oxygen consumption rate was calculated from the maximum slope in the plot of [O_2] versus time.

Electronic spectra of reactions of ferulic and caffeic acids with H_2O_2, catalyzed by PGH synthase or horseradish peroxidase, were recorded using a

Cary 219 spectrophotometer in the 220-380 nm region where both compounds absorb strongly. Both reactions with excess H_2O_2, catalyzed by PGH synthase, were incomplete. Upon addition of more enzyme the reactions could be driven to completion. For completed reaction the absorbances of the caffeic acid-product at 310 nm were determined for a series of [caffeic acid] and the molar absorptivity of the caffeic acid product, $\varepsilon_{CAF,\ pr} = 5.12 \pm 0.17\ mM^{-1}cm^{-1}$, was obtained. The molar absorptivity for pure caffeic acid was then corrected for this value, and a $\Delta\varepsilon_{CAF} = 7.35\ mM^{-1}cm^{-1}$ was then used in the initial rate calculations.

Steady-state experiments were performed with a Union Giken RA-601 stopped flow apparatus. The changes in absorbance at 310 nm due to the reaction of caffeic acid with PGH synthase and H_2O_2 as a function of time were measured using constant nM concentration of enzyme, and varying and high concentration of H_2O_2. After conversion of the absorbance into concentration, the initial rates of disappearance of caffeic acid,-d[CAF]/dt, were determined. The spontaneous decay of caffeic acid (no hydrogen peroxide was present) was within the experimental error and the initial rates were not corrected for those values. Curve fittings and calculations were made using a non-linear regression data analysis program Enzfitter, Elsevier-Biosoft.

3.3. RESULTS

3.3.1. The Stimulation of PGH Synthase Cyclooxygenase Activity by Ferulic and Caffeic Acids

Both ferulic and caffeic acids enhance the oxidation of arachidonic acid catalyzed by PGH synthase (Figure 3.1). Under identical experimental conditions, ferulic acid showed higher capacity to stimulate cyclooxygenase activity than caffeic acid. The concentrations of ferulic and caffeic acids necessary to produce

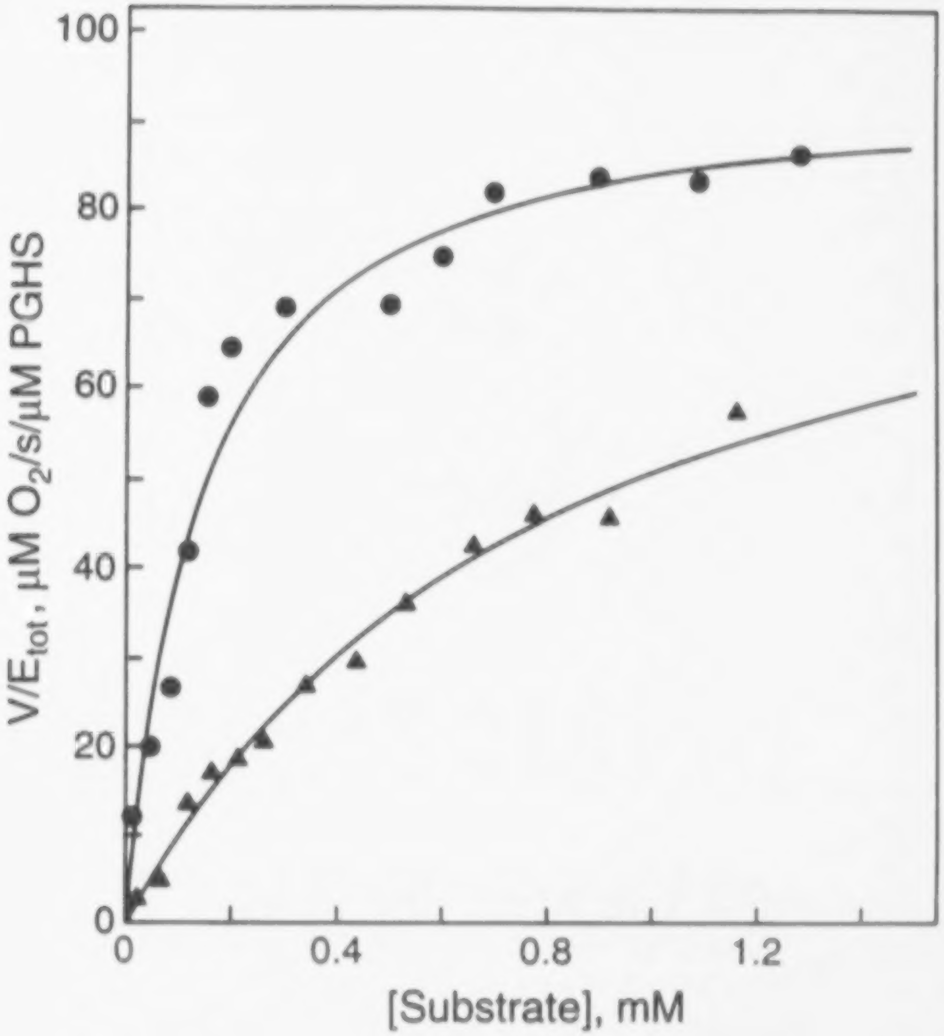

Figure 3. 1. The stimulation of PGH synthase cyclooxygenase activity by ferulic and caffeic acids. Plots of v/E$_{tot}$ versus [ferulic acid] (●) or [caffeic acid] (▲) at 30 ±1°C in 0.1 M phosphate buffer (pH 8.0) with 0.2 mM arachidonic acid, 20 nm enzyme and without hematin.

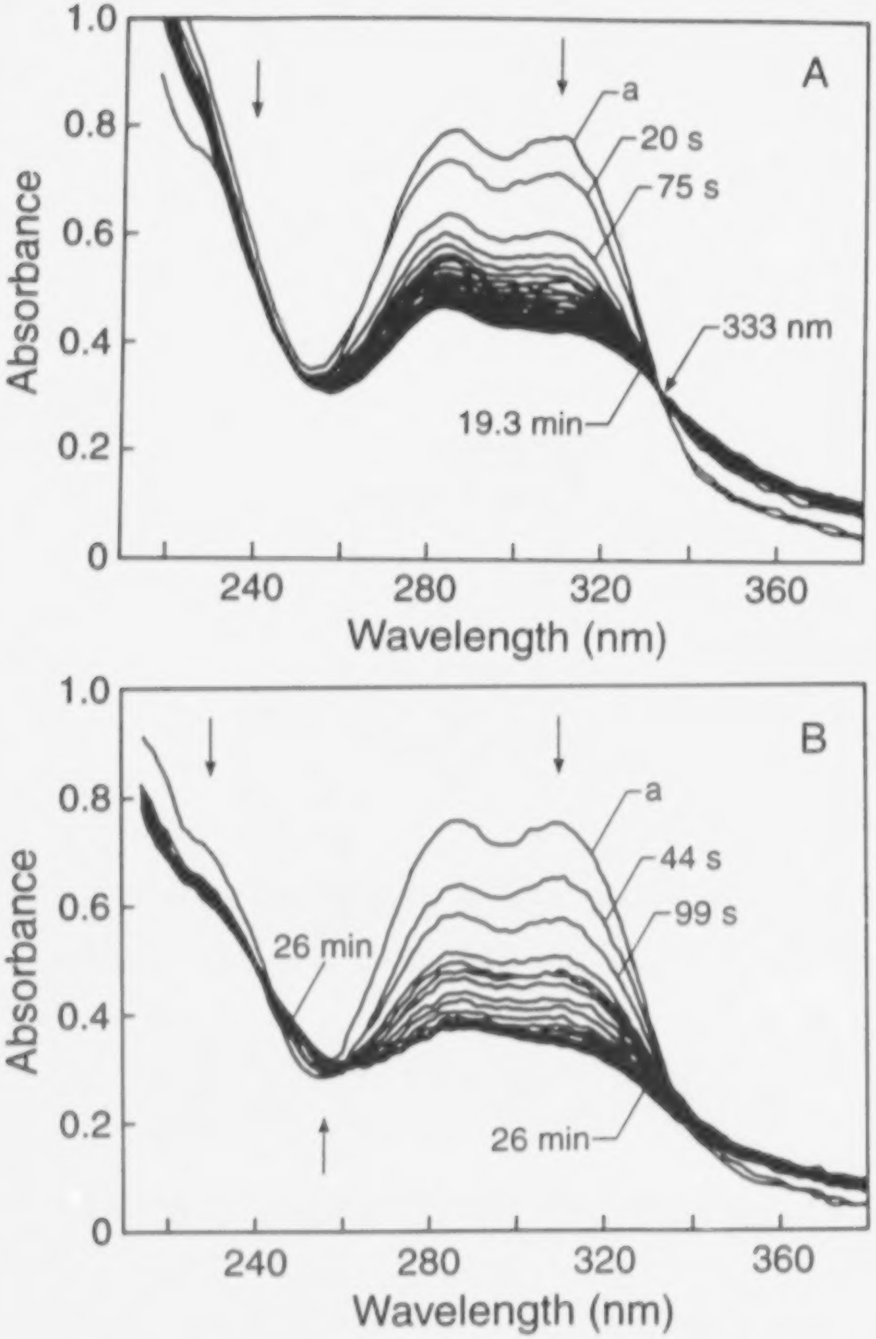

Figure 3. 2. Changes in electronic spectrum of ferulic acid (48.5 μM) during its oxidation with **(A)** 0.265 mM H_2O_2 and 53.9 nM PGH synthase or **(B)** 0.265 mM H_2O_2 and 12.4 nM horseradish peroxidase at 25 ± 1°C in 0.1 M phosphate buffer (pH 8.0).

50 % stimulation (A_{50}) are 0.14 ± 0.02 and 0.8 ± 0.1 mM respectively, when 0.2 mM arachidonic acid and 20 nM enzyme were used. On the other hand, maximum velocities for oxygen uptake did not differ significantly for the two substrates; k_{cat} values in the presence of ferulic or caffeic acids are 95 ± 4 and 92 ± 3 s^{-1}, respectively. The measurements above 1.5 mM of ferulic or caffeic acid were limited by their low solubility in 0.1 phosphate buffer, pH 8.0, so that it is not known whether they stimulate or inhibit the prostaglandin biosynthesis at higher concentrations.

3.3.2. Spectral Changes during the Steady-State Oxidation of Ferulic Acid by H_2O_2 Catalyzed by Horseradish Peroxidase or PGH Synthase

Ferulic acid strongly absorbs in the UV region showing two maxima, at 286 and 310 nm in a phosphate buffer, pH 8.0 (Figure 3.2). In the presence of an excess of H_2O_2 and a catalytic amount of either horseradish peroxidase or PGH synthase, ferulic acid is oxidized very rapidly but in a different way, depending on which enzyme is used. In the reaction catalyzed by horseradish peroxidase, a mixture of products is present from the early stages of the reaction (Figure 3.2B). In the case of PGH synthase catalysis, a single product of ferulic acid is formed. This is ascertained from the existence of an isosbestic point between ferulic acid and the ferulic acid-product at 333 nm (Figure 3.2A).The reaction shown is 85% complete. A subsequent addition of PGH synthase caused an additional drop in absorbance (data not shown). The enzyme and H_2O_2 were added once more, but did not cause further changes in the ferulic acid-product spectrum. However, when horseradish peroxidase was added to the same sample, obtained from the PGH synthase-catalyzed reaction, small changes in the ferulic acid-product spectrum were observed; the isosbestic point at 333 nm was lost and the spectrum was more like one observed in the reaction of ferulic acid with horseradish peroxidase and H_2O_2 (data not shown).

3.3.3. Spectral Changes During the Steady-state Oxidation of Caffeic Acid by H_2O_2 Catalyzed by Horseradish Peroxidase or PGH Synthase

The shape of the spectrum and the absorptivity of caffeic acid in the UV region are very similar to ferulic acid (Figure 3.3). Caffeic acid shows two maxima, at 286 and 312 nm in phosphate buffer, pH 8.0. Caffeic acid is a good reducing substrate for both horseradish peroxidase and PGH synthase, but significant differences exist in the two peroxidatic reactions of caffeic acid. For horseradish peroxidase, caffeic acid undergoes oxidation to a complex mixture of products. For clarity, only initial steps of the reaction are shown in Figure 3.3B. Further reaction, probably polymerization of the caffeic acid products, occurred in later stages.

In PGH synthase catalysis (Figure 3.3A), caffeic acid is initially (3.8 min) transformed to a product having a strong maximum at 247 nm and a weak, broad band in the 266-320 nm region. During the next 22 min a second product is formed, having an isosbestic point with the first product at 262 nm and a maximum at 275 nm. The second product does not undergo any further transformation.

3.3.4. Steady-State Kinetics for the Oxidation of Caffeic Acid with H_2O_2 Catalyzed by PGH Synthase

Representative stopped flow kinetic traces obtained at a fixed [caffeic acid] and at three different concentrations of H_2O_2 are shown in Figure 3.4. At higher

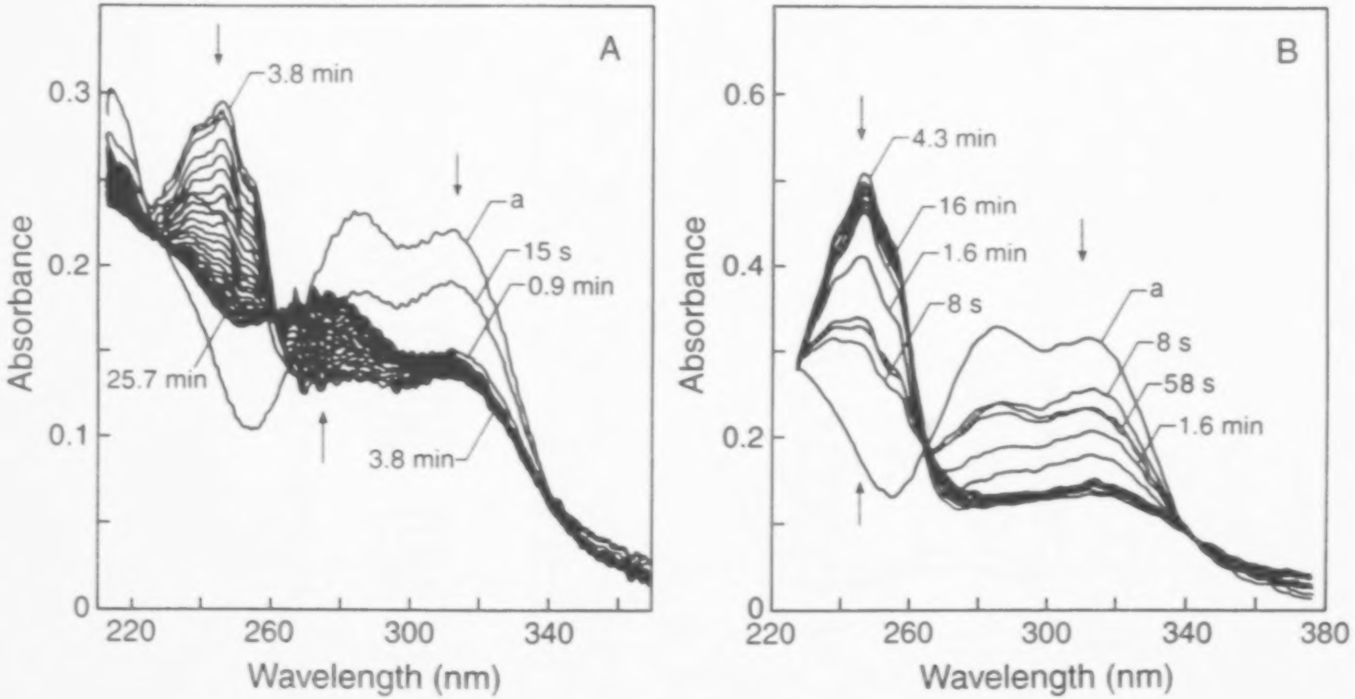

Figure 3.3. Spectral changes of caffeic acid (17.5 µM) during its oxidation with **(A)** 0.488 mM H_2O_2 and 50.76 nM PGH synthase, or **(B)** 25.2 µM caffeic acid with 0.244 mM H_2O_2 and 12.6 nM horseradish peroxidase in 0.1 M phosphate buffer, pH 8.0, and at 25 ± 1°C.

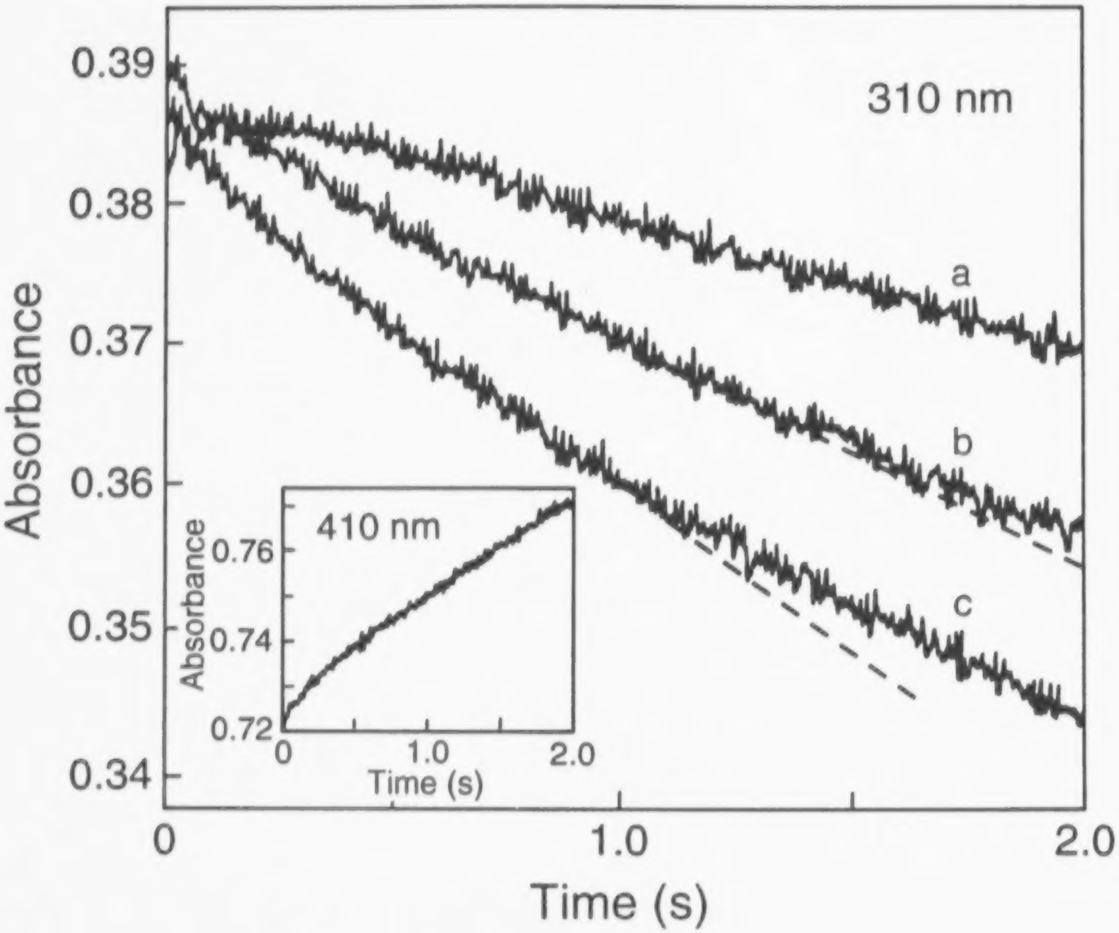

Figure 3. 4. Kinetic traces of caffeic acid (50.1 μM) at 310 nm after its reaction with 25 nM enzyme and 0.372 (a), 0.743 (b), and 1.67 (c) mM H_2O_2 in 0.1 phosphate buffer, pH 8.0, at 25 ± 0.5°C. *Inset:* Change in absorbance at 410 nm caused by PGH synthase-compound II reduction to the native enzyme by caffeic acid. The reaction mixture contained 10 μM caffeic acid, 0.79 mM H_2O_2 and 0.126 μM enzyme.

Table 3.1 Steady-state kinetic parameters for the peroxidase reaction of PGH synthase with caffeic acid and H_2O_2[a]

Caffeic Acid [b] (μM)	k_{cat}^{app} (s -1)	K_m^{app} (mM)
10.2	20.2 ± 0.5[c]	0.025 ± 0.008
20.2	46.8 ± 0.6	0.06 ± 0.01
50.0	126 ± 2	0.27 ± 0.02
75.8	195 ± 2	0.56 ± 0.04
100.2	239 ± 2	0.82 ± 0.02

[a] Data were obtained from plotting the initial rate of caffeic acid disappearance per unit enzyme concentration ($v/[E]_{tot}$) against [H_2O_2] at fixed caffeic acid concentrations. All measurements were performed in 0.1 M phosphate buffer, pH 8.0, at 25 ± 0.5 °C; [b] Parameters k_{cat}^{app} and K_m^{app} were obtained after fitting the Michaelis -Menten equation to the experimental data; [c] Standard deviation.

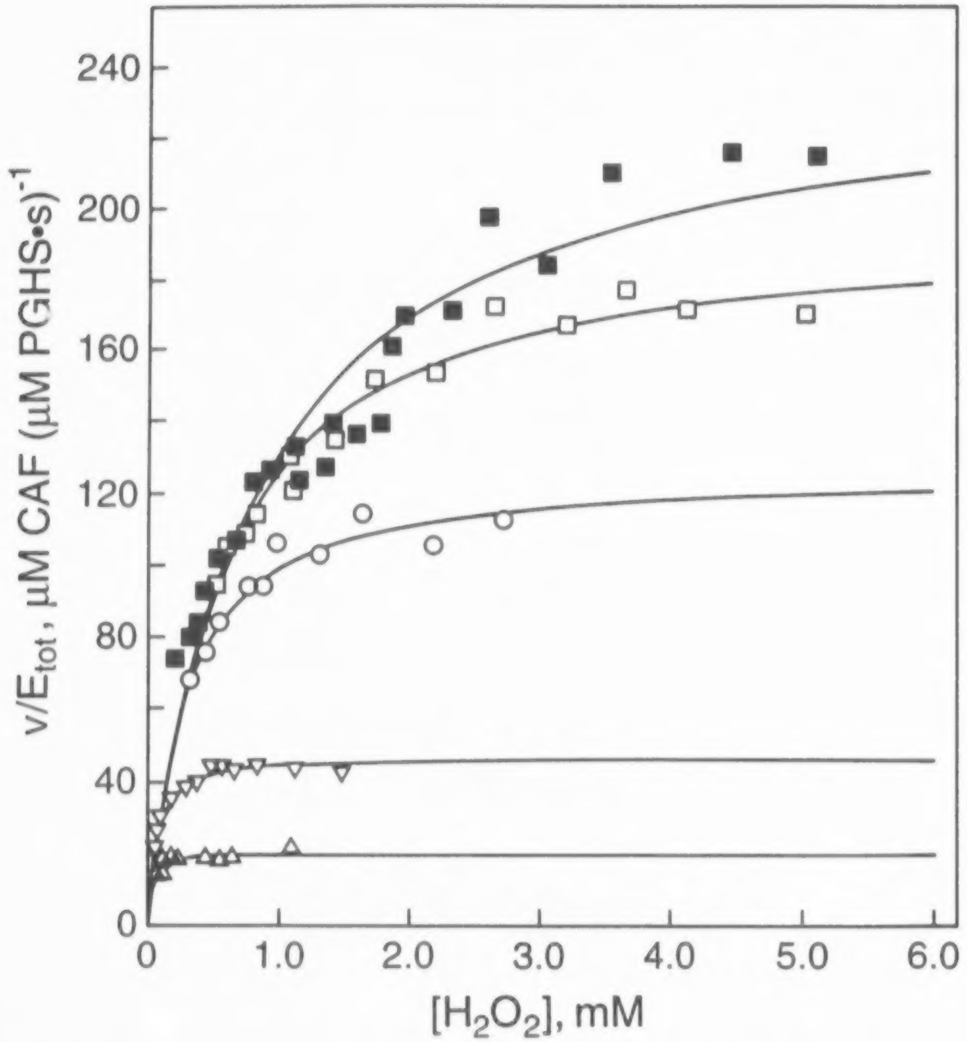

Figure 3. 5. Initial rates at unit total enzyme concentration of the oxidation of the caffeic acid obtained for the series of [caffeic acid]. [Caffeic acid] are 10.2 (Δ), 20.2 (∇), 50.0 (O), 75.8 (□), and 100.2 (■) μM. The enzyme concentration is 32.5 nM for all experiments except for (Δ), where it is 29.6 nM.

reaction rates departures from linearity are occurring earlier (compare traces *a*, *b* and *c in* Figure 3.4). However, linear traces were almost always obtained over the 0.5-1.0 s time scale for a broad range of [caffeic acid] and [H_2O_2]. The reaction of PGH synthase-compound II with caffeic acid is the rate limiting step in the enzymatic cycle under our experimental conditions. A steady reduction of PGH synthase-compound II to the native enzyme by caffeic acid was observed at 410 nm (Figure 3.4, inset). A similar trace for PGH synthase-compound II formation at 426 nm was observed but is not shown.

Applying Beer's law and using $\Delta\varepsilon_{CAF}$, the initial rates,$-d[CAF]/dt$, were calculated and plotted as a function of [H_2O_2] at fixed [caffeic acid]. A series of saturation curves were obtained. The results are summarized in Table 3.1 and Figure 3.5. A plot of k_{cat}^{app} versus K_m^{app} (data from Table 3.1) is not linear, as would have been expected for the irreversible ping-pong mechanism. Rather there is a decrease in the k_{cat}^{app}/K_m^{app} ratio when [caffeic acid] is increased since K_m^{app} increased faster than predicted; the values of k_{cat}^{app} were linearly dependent on [caffeic acid] as in the irreversible ping-pong mechanism. We conclude that caffeic acid exerts an inhibitory effect on the overall reaction and propose a mechanism in which a reversible, non-productive binding of caffeic acid to the native enzyme (eq. 3.1, with dissociation constant K_D) is followed by the irreversible ping-pong reaction mechanism (eqs. 3.2-3.4):

$$\mathrm{PGHS + CAF} \overset{K_D}{\rightleftharpoons} \mathrm{PGHSCAF} \qquad [3.1]$$

$$\mathrm{PGHS + H_2O_2} \xrightarrow{k_1} \mathrm{PGHS\text{-}I} \qquad [3.2]$$

$$\mathrm{PGHS\text{-}I + CAF} \xrightarrow{k_2} \mathrm{PGHS\text{-}II + CAF\cdot} \qquad [3.3]$$

$$\mathrm{PGHS\text{-}II + CAF} \xrightarrow{k_3} \mathrm{PGHS + CAF\cdot} \qquad [3.4]$$

The corresponding rate law and relevant equations (Appendix A.2) are:

$$\frac{-d[CAF]/dt}{[PGHS]_{tot}} = \frac{k_{cat}^{app}\ [H_2O_2]}{K_m^{app} + [H_2O_2]} \qquad [3.5]$$

$$k_{cat}^{app} = \frac{2\ k_1\ k_2\ k_3\ [CAF]}{k_1\ (k_2 + k_3)} \qquad [3.6]$$

$$K_m^{app} = \frac{k_2 k_3\ [CAF]\ (1 + [CAF]/\ K_D)}{k_1\ (k_2 + k_3)} \qquad [3.7]$$

$$\frac{k_{cat}^{app}}{K_m^{app}} = \frac{2\ k_1}{1\ + [CAF]/K_D} \qquad [3.8]$$

Caffeic acid therefore acts as inhibitor of its own oxidation by enzyme higher oxidation states.

The above mechanism predicts that k_1, k_3 and K_D can be determined. Under our experimental conditions, the reaction of PGH synthase-compound II with caffeic acid is the rate controlling step. Then, $k_2 >> k_3$ and eq.3.6 is simplified to eq. 3.9:

$$k_{cat}^{app} = 2\ k_3\ [CAF] \qquad [3.9]$$

From eq. 3.8, a double reciprocal plot of the form

$$K_m^{app}/k_{cat}^{app} = 1/2\ k_1 + 1/(2\ k_1 K_D) x\ [CAF] \qquad [3.10]$$

is obtained.

Our results satisfy the conditions of eqs. 3.9 and 3.10 (i) k_{cat}^{app} is a linear function of [caffeic acid] and the slope is the rate constant for the reaction of PGH synthase-compound II with caffeic acid, i.e., $k_3 = (1.25 \pm 0.1) \times 10^6\ M^{-1}s^{-1}$;

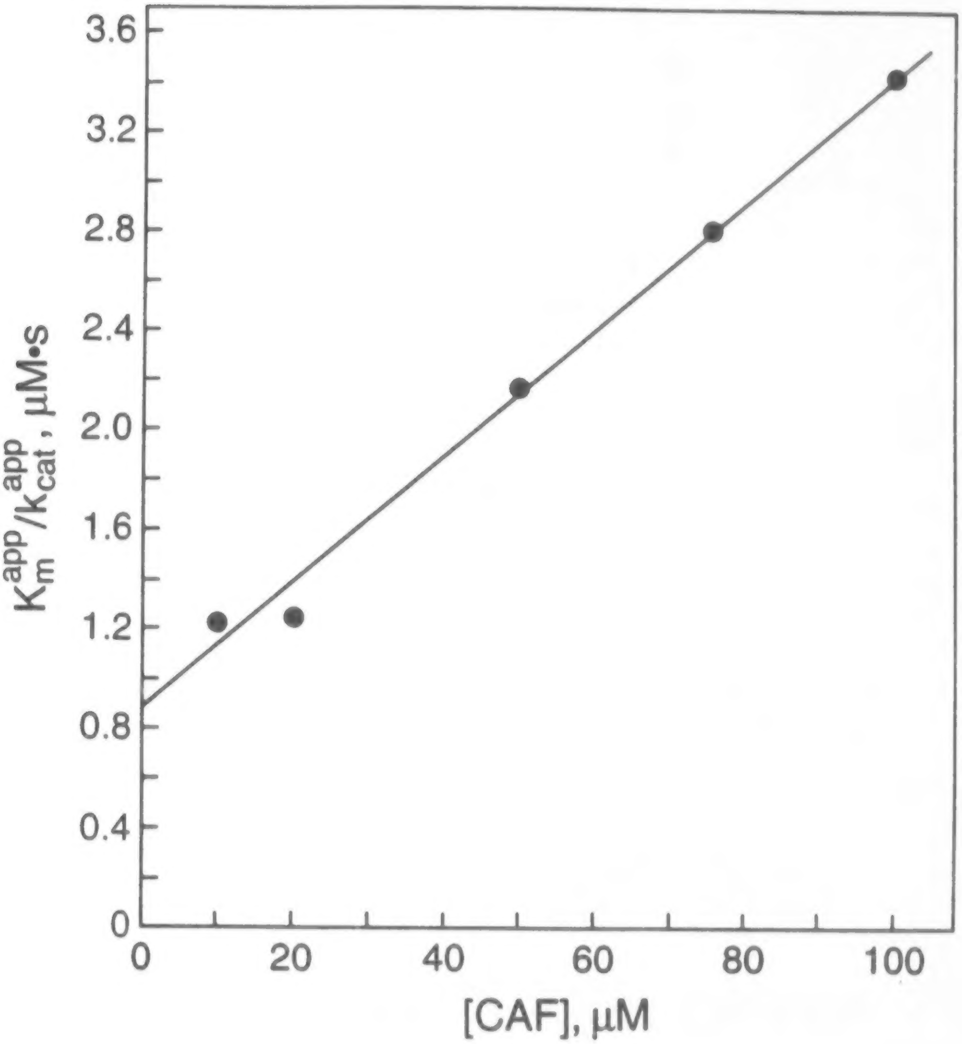

Figure 3. 6. Linear dependence of K_m^{app}/k_{cat}^{app} versus [caffeic acid], according to the modified irreversible ping-pong peroxidatic mechanism summarized in eqs. 3.1-3.10.

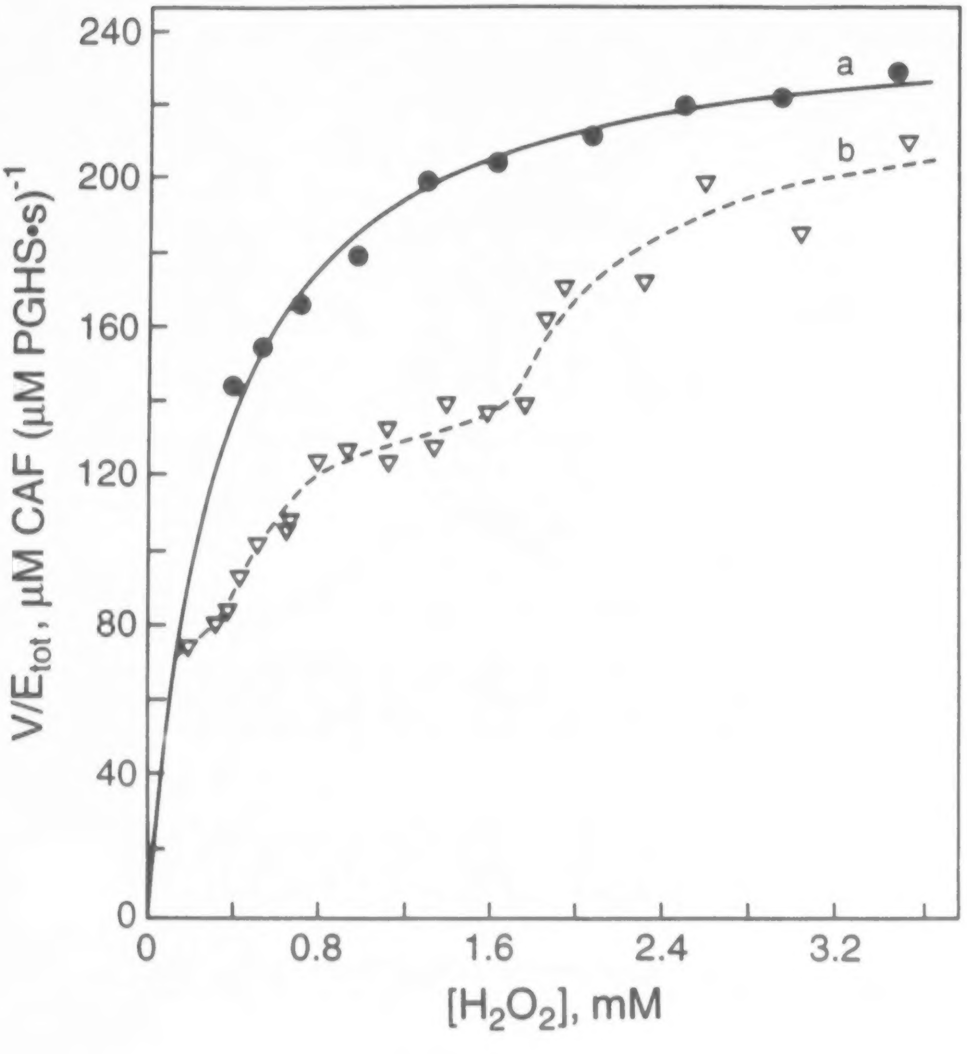

Figure 3. 7. The effect of PGH synthase concentration on the oxidation of caffeic acid. Upper curve : 100 µM caffeic acid, and 16.2 nM enzyme. Lower curve : 100 µM caffeic acid, and 32.4 nM enzyme.

(ii) K_m^{app}/k_{cat}^{app} is also a linear function of [caffeic acid] (Figure 3.6); from the intercept and the slope of the curve, the rate constant for PGH synthase-compound I formation, $k_1 = (5.7 \pm 0.1) \times 10^5\ M^{-1}s^{-1}$, and the dissociation constant for the inhibitory complex, $K_D = 35 \pm 10\ \mu M$, were determined.

An unexpected biphasic behavior was observed when 100 μM CAF reacted with H_2O_2 and 32.4 nM enzyme (Figure 3.7, lower curve). At half the enzyme concentration, 16.2 nM, a normal saturation curve was observed (Figure 3.7, upper curve). The biphasic curve could approximately be fitted to two consecutive rectangular hyperbolae. These yield two sets of k_{cat}^{app} and K_m^{app} values, labelled α and β, for the initial and latter parts of the curve. Applying eqs. 3.9 and 3.10, and assuming that $k_1 = 10^6\ M^{-1}s^{-1}$, we estimate that $k_3{}^{\alpha} : k_3{}^{\beta} = 0.6$, and $K_D{}^{\alpha} : K_D{}^{\beta} = 5.0$. This indicates that caffeic acid is oxidized faster (higher k_3) and has a higher binding affinity (lower K_D) at one site or subunit (β) than on another site or subunit (α).

It should be noted that the kinetic data summarized in Table 3.1 represent the average values of two saturation curves; therefore, the k_1, k_3 and K_D are the net kinetic constants for two types of interaction of caffeic acid and PGH synthase.

3.4 DISCUSSION

3.4.1. Comparison of the Catechol Derivative Caffeic Acid with Structurally Related Phenol Derivatives in their Stimulation of Prostaglandin Biosynthesis

Our oxygen consumption measurements showed that both substrates caffeic and ferulic acid prevent non-enzymatic oxidation of arachidonic acid initiated with H_2O_2 by acting as classical chain-breaking antioxidants (data not shown). In contrast, in the presence of PGH synthase the two substrates stimulate arachidonic acid oxygenation. The effectiveness of stimulation of arachidonic acid oxidation is in the order: ferulic acid >*p*-coumaric acid > caffeic acid (Table 3.2). Phenols are better stimulators of prostaglandin biosynthesis than catechols. Furthermore, ferulic acid is a better stimulator than *p*-coumaric acid, in agreement with their reduction potentials and rate constants for their reactions with PGH synthase compound II. Unexpectedly, caffeic acid is the best reducing agent in the series but provided the least stimulation of the cyclooxygenase activity. It suggests that caffeic acid, a catechol, influences the enzyme differently than ferulic and *p*-coumaric acids which are phenols. This study shows that caffeic acid has a different peroxidatic mechanism than phenols; it interferes with the H_2O_2 reaction (PGH synthase compound I formation) which is then reflected in the overall cyclooxygenase reaction.

3.4.2. Comparison of PGH Synthase and Horseradish Peroxidase Reactions and Evidence Against O-Demethylation Mechanism

In studying the peroxidatic reaction mechanism involving ferulic and caffeic acids, we first made a comparison of the PGH synthase and horseradish peroxidase reactions in terms of enzyme intermediates and products formed under steady state conditions.

During ferulic acid oxidation by horseradish peroxidase and H_2O_2 no isosbestic point was detected. During ferulic acid oxidation by PGH synthase and H_2O_2 an isosbestic point is formed (Figure 3.2). However, after two subsequent additions of H_2O_2 and PGH synthase, the ferulic acid final spectrum

Table 3. 2. Effects of structure on the ability of cinnamic acid derivatives to act as stimulators of cyclooxygenase and reducing substrates of PGH synthase peroxidase

Compound	A_{50} [a] (mM)	k_{cat} (s^{-1})	k_1 ($10^5\ M^{-1}s^{-1}$)	k_3 ($10^6\ M^{-1}s^{-1}$)	K_D (μM)	E^o, [b] (V)
Caffeic Acid (3,4-dihydroxycinnamic acid)						
	0.8 ± 0.1	92 ± 3	5.68 ± 0.07	1.25 ± 0.1	35 ±10	+ 0.35
Ferulic Acid (3-methoxy-4-hydroxycinnamic acid)[c]						
	0.14 ± 0.02	95 ± 4	6.43 ± 0.03	2.77 ± 0.30	NB [d]	+ 0.57
p-Coumaric Acid (4-hydroxycinnamic acid)[e]						
	0.35 ± 0.03	87 ± 3	4.6 ± 0.1	1.27 ± 0.04	NB	+ 0.73

---

[a] For definitions and calculations of A_{50}, k_{cat}, k_1, k_3 and K_D see Results.

[b] Standard potentials, versus Ag/AgCl at 200 mV/s in 1M acetate buffer, pH 4.7 (ref. 30)

[c] Ref. 31 and Chapter 4.

[d] No binding

[e] Ref. 20 and Capter 2

was similar to the final ferulic acid-product spectrum in the horseradish peroxidase catalyzed reaction, which is well established not to be a cytochrome P-450 type of reaction (14). This was unexpected, since an O-demethylating mechanism of PGH synthase reaction with ferulic acid yielding caffeic acid, similarly to cytochrome P-450 mechanism, was suggested for a rat lung homogenate/O_2/arachidonic acid system (14). Additional evidence indicates that the purified enzyme does not transform the phenol to catechol and that consequently PGH synthase does not act as an oxygenase but as peroxidase in this reaction:

(i) caffeic acid is a reducing substrate for both enzymes, horseradish peroxidase and PGH synthase. Its oxidation spectra are distinct from the spectra of the ferulic acid oxidation with these two enzymes (Figs. 3.2 and 3.3);

(ii) the single isosbestic point observed during ferulic acid oxidation with PGH synthase and hydrogen peroxide (Figure 3.2A) would not be observable if caffeic acid were the product of the oxidation; caffeic acid would be oxidized simultaneously in this system;

(iii) an incomplete oxidation of ferulic acid with PGH synthase and hydrogen peroxide can be completed by subsequent addition of horseradish peroxidase; the spectra of products resemble the ferulic acid oxidation (Figure 3.2B) not the caffeic acid oxidation (Figure 3.3B) with the horseradish peroxidase.

It appears that PGH synthase catalyzes ferulic and caffeic acids peroxidation more specifically than horseradish peroxidase. PGH synthase probably controls the free radical liberation rather than unleashing an uncontrolled chain reaction as in the case of horseradish peroxidase.

3.4.3. The Effect of Structure on the Binding and Oxidation of Catechol and Phenol Cinnamic Acid Derivatives by PGH Synthase

The results of the steady-state kinetic measurements reveal that caffeic acid is oxidized by PGH synthase higher oxidation state intermediates through the irreversible ping-pong mechanism as is the case for phenolic compounds (2, 20) but for caffeic acid this pathway is modified by its non-productive binding to the native enzyme.The rate constants for the peroxidase reaction with PGH synthase-compound II, k_3, are not in the order that would be expected from the structures and electronic properties of these derivatives (Table 3.2). The electron-releasing property of the methoxy group increases the ease of oxidation of ferulic acid and increases the reaction rate from that previously established for *p*-coumaric acid (20). This is as expected, since it is known that electronic but not hydrophobic properties of monophenols determine the rates of their reactions with PGH synthase-compound II (2).

The second hydroxyl group in caffeic acid does not contribute significantly to the reaction rate with PGH synthase-compound II, but contributes to the inhibitory binding to the enzyme. The binding of caffeic acid is fast and reversible. It is related to the specific structural feature of the caffeic acid, i.e, the presence of two adjacent hydroxyl groups. Binding to the native enzyme did not occur when the second hydroxyl group of caffeic acid was replaced with a methoxy group (ferulic acid) or was absent (*p*-coumaric acid) (Table 3.2). Caffeic acid is not coordinated to the heme iron. We did not observe any changes in the enzyme spectra in the Soret region, but we did observe minor changes in the caffeic acid UV spectra after enzyme addition. Furthermore, caffeic acid did not inhibit cyclooxygenase activity and probably did not interfere with arachidonic acid binding.

3.4.4. The Nature of the Caffeic Acid Binding to PGH Synthase

The mechanism for the reaction of caffeic acid with PGH synthase and H_2O_2 has been deduced from steady-state kinetic measurements in the range of 10-100 μM caffeic acid. Biphasic behaviour is seen at 100 μM caffeic acid and higher enzyme concentrations (Figure 3.7b). Such biphasic curves indicate that two catalytic events are occurring that can be partially separated. They can be explained by either the existence of two distinct and independent binding sites for caffeic acid or from a cooperation of the two enzyme subunits in the binding and oxidation of caffeic acid.

Several studies indicate that some conformational changes could be involved in the regulation of PGH synthase activity and its sensitivity to proteolysis (21-26). Only the Arg^{235} region is available for proteolytic attack in the apoenzyme. Heme binding makes this region more inaccessible to trypsin cleavage (21, 22). Cyclooxygenase inhibitors also produce resistance to proteolysis (23) and stabilize the peroxidase activity against extremes of temperature and pH (24). Published data for the stoichiometry of the heme and nonsteroidal antiinflammatory drugs binding to PGH synthase vary (25, 26), but 1 mol/mol of subunit for both is generally accepted (27). A stoichiometry lower than 1 mol of heme/mol of subunit is usually obtained in enzyme preparations (28). As was previously suggested (23), the lower stoichiometry may be the result of a negative allosteric interaction between the two enzyme subunits, but this has not been proven experimentally.

Our kinetic study also indicates that PGH synthase-peroxidase can act either as an allosteric enzyme or that there is non-equivalent binding for caffeic acid. From the reaction of caffeic acid it is tempting to suggest that negative cooperativity between two enzyme subunits is more probable than the binding to two independent non-equivalent sites. The two PGH synthase subunits are of similar structure (27); caffeic acid could be probably equally efficiently bound to both subunits. If the binding were independent then two binding constants of similar magnitude would be expected and a biphasic curve would not be observed. It appears more probable that caffeic acid after binding changes the arrangement and relation between the enzyme subunits so that they appear to have different affinity and capacity for catalysis. The observed perturbations in the reaction rates and affinity (Figure 3.7, b) could be due to the effect of one subunit on the activity of the other.

It is not known whether the enzyme can function as a monomer (27), but at high dilution a dissociation into two subunits is likely. The upper curve in Figure 3.7 could be that of the enzyme monomer or at least of a partially dissociated dimer. Enzyme dilution led to loss of biphasic behavior; the enzyme reactivity (k_{cat}) is higher and the caffeic acid affinity for the enzyme (K_m) is lower than what was observed at higher enzyme concentration.

There is always doubt however, when behavior such as in our study is observed, that an enzyme having different reactivity was also present. This possibility is very unlikely since we used 87% holoenzyme of high specific activity and our technique measures reactivity of the holoenzyme only. Moreover, there is no evidence for different isoenzymes of PGH synthase isolated from sheep seminal vesicles (27, 29)

3.4.5. Conclusion

Our results demonstrate that the naturally occurring catechol derivative, caffeic acid, can be involved in prostaglandin biosynthesis as a good reducing substrate of PGH synthase-peroxidase and a moderate stimulator of PGH synthase-cyclooxygenase activity. A practical aspect of this study is the additional finding that caffeic acid can affect yet another pathway in the arachidonic acid cascade. It is known that caffeic acid inhibits 5- and 12-lipoxygenase pathways by reducing active site iron as do many other lypooxygenase inhibitors (10, 11). Our study showed that this lipoxygenase inhibitor stimulates PGH synthase-cyclooxygenase, serving as a reducing substrate for PGH synthase-peroxidase. Consequently good inhibitors of leucotriene biosynthesis potentiate prostaglandin biosynthesis, acting as reducing agents in both cases. It suggests that structurally similar catechols may possess therapeutic effects different to conventional non-steroidal anti-inflammatory drugs. For the latter class of drugs it is believed that they usually modulate only one of the pathways in arachidonic acid metabolism.

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CHAPTER FOUR

INTIMATE RELATION BETWEEN CYCLOOXYGENASE AND PEROXIDASE ACTIVITIES OF PROSTAGLANDIN ENDOPEROXIDE SYNTHASE. PEROXIDASE REACTION OF FERULIC ACID AND ITS INFLUENCE ON THE REACTION OF ARACHIDONIC ACID[*]

4.1. INTRODUCTION

In this chapter we study the mutual relationship of the peroxidase and cyclooxygenase functions of prostaglandin endoperoxide synthase (PGH synthase)[**]. We showed in Chapter Three that ferulic acid strongly stimulates the oxygenation of arachidonic acid. At the same time, ferulic acid was not oxidized to caffeic acid as it was claimed previously (1). This would be a cytochrome P-450 not peroxidase type of the reaction of PGH synthase. In this chapter we establish that ferulic acid serves as a classical reducing substrate for PGH synthase-peroxidase and that this reaction obeys the irreversible ping-pong kinetics as in the case of structurally related *p*-coumaric acid (Chapter Two).

The establishment of the peroxidatic reaction mechanism of ferulic acid with PGH synthase is essential before examining the effect of ferulic acid on the reaction of arachidonic acid. To see the relationship between the peroxidase and cyclooxygenase functions of PGH synthase we compare the rates of the simultaneous disappearence of ferulic acid and arachidonic acid in the reaction mixtures using different concentration ratios of the two compounds.

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[**] Abbreviations: PGH synthase, prostaglandin endoperoxide synthase (EC 1.14.99.1); Compound-I'-Tyr., the tyrosyl radical form of compound I of PGH synthase formed by internal electron transfer; HAA, arachidonic acid; · AA, free radical derived by hydrogen atom abstraction of the 13-*pro-S* hydrogen atom from AA; PGG_2, the hydroperoxide prostaglandin G_2; $\cdot$.PGG_2, the hydroperoxyl radical of PGG_2; PGH_2, prostaglandin H_2, the alcohol obtained by reduction of PGG_2; HOPh, phenol reducing substrate, in this study ferulic acid; ·OPh, free radical derived by hydrogen atom abstraction from ferulic acid; DDC, diethyldithiocarbamate; ε, molar absorptivity. Subscripts to ε: FA, ferulic acid; FA pr, product of ferulic acid oxidation; PGHS, prostaglandin H synthase. $\Delta\varepsilon_{FA}$, difference in molar absorptivity between ferulic acid and its oxidation product.

We examine two mechanisms which it has been claimed account for the experimental observations when both arachidonic acid and a reducing substrate are present. The first is the tyrosyl radical chain mechanism (2, 3):

$$\text{Initiation}\left\{\begin{array}{rcll} \text{PGH Synthase} + \text{PGG}_2 & \rightarrow & \text{Compound I} + \text{PGH}_2 & [4.1] \\ \text{Compound I} & \rightarrow & \text{Compound-I'-Tyr}\cdot & [4.2] \end{array}\right.$$

$$\text{Propagation}\left\{\begin{array}{rcll} \text{Cpd-I'-Tyr}\cdot + \text{HAA} & \rightarrow & \text{Compound II} + \cdot\text{AA} & [4.3] \\ 2\text{O}_2 + \cdot\text{AA} & \rightarrow & \cdot\text{PGG}_2 & [4.4] \\ \text{Compound II} + \cdot\text{PGG}_2 & \rightarrow & \text{Compound-I'-Tyr}\cdot + \text{PGG}_2 & [4.5] \end{array}\right.$$

$$\text{Termination}\left\{\begin{array}{rcll} \text{Compound I} + \text{HOPh} & \rightarrow & \text{Compound II} + \cdot\text{OPh} & [4.6] \\ \text{Compound II} + \text{HOPh} & \rightarrow & \text{PGH Synthase} + \cdot\text{OPh} & [4.7] \end{array}\right.$$

According to the above mechanism, the initiation steps consist of compound I formation (eq. 4.1); the compound I converts spontaneously to a tyrosyl radical species containing the same total number of oxidizing equivalents (eq. 4.2). The tyrosyl radical can be regenerated many times in the propagation steps (eqs. 4.3-4.5). The phenol radicals $\cdot$OPh, formed in eqs. 4.6 and 4.7, are not scavenged by molecular oxygen; by reacting with each other they would be chain terminators in the above mechanism (4). No connection between the rates of arachidonic acid and ferulic acid disappearance would be expected for different starting concentrations of the two substrates. Furthermore, the tyrosyl radical chain mechanism would be inhibited by added phenols in two ways: phenols, by competing for compound I, would inhibit the initiation steps, and by reacting with compound II would also interfere with the propagation steps. Thus with increasing phenol concentration the ratio of phenol/arachidonic acid reacted would also be expected to increase.

The alternative mechanism is one which does not involve a branched chain reaction but rather a normal peroxidatic reaction cycle in which one of the steps is abstraction of the 13-*pro-S* hydrogen atom from arachidonic acid by the typical peroxidase compound I, a $Fe^{IV}{=}O$ porphyrin π-cation radical (5):

$$\text{PGH Synthase} + \text{PGG}_2 \rightarrow \text{Compound I} + \text{PGH}_2 \qquad [4.1]$$

$$\text{Compound I} + \text{HAA} \rightarrow \text{Compound II} + \cdot\text{AA} \qquad [4.2]$$

$$2\text{O}_2 + \cdot\text{AA} \rightarrow \cdot\text{PGG}_2 \qquad [4.3]$$

$$\text{HOPh} + \cdot\text{PGG}_2 \rightarrow \text{PGG}_2 + \cdot\text{OPh} \qquad [4.4]$$

$$\text{Compound II} + \text{HOPh} \rightarrow \text{PGH Synthase} + \cdot\text{OPh} \qquad [4.5]$$

The sum of these reactions is

$$\text{HAA} + 2\text{HOPh} + 2\text{O}_2 \rightarrow \text{PGH}_2 + 2\cdot\text{OPh} \qquad [4.6]$$

The above peroxidase mechanism (arachidonic acid reacting with the classical compound I and the phenol, ferulic acid reacting with compound II), leads to the prediction of a fixed 2:1 ratio of ferulic acid/arachidonic acid reacted, and this is what we have observed and report upon in this chapter.

4.2. MATERIALS AND METHODS

4.2.1. Materials

PGH synthase was purified from ram seminal vesicle microsomes (Chapter Two; 6, 7). The isolated enzyme was a 40/60 mixture of holo- and apoenzyme having a specific cyclooxygenase activity of 37 μM arachidonic acid (mg protein-min)$^{-1}$ in the presence of 1 μM hematin and 1 mM phenol. Enzyme concentrations were assayed spectrophotometrically using an ε_{PGHS} of 123 mM^{-1} cm^{-1} at 410 nm (8) and protein content by the BioRad protein assay using bovine serum albumin as a standard.

Arachidonic and ferulic acids and hematin were obtained from Sigma, and H_2O_2 from Fisher. Arachidonic acid was dissolved in deaerated 98% ethanol solution and kept in small portions under nitrogen at -70°C. Ferulic acid was recrystallized from water. The concentration of H_2O_2 was determined spectrophotometrically using $\varepsilon_{H_2O_2} = 43.6\ M^{-1}cm^{-1}$ at 240 nm (9).

Curve fittings and calculations on all our results were made using a non-linear regression data analysis program by Enzfitter, Elsevier-Biosoft.

4.2.2. Oxidation of Ferulic Acid by H_2O_2 Catalyzed by PGH Synthase

A Union Giken RA-601 stopped flow apparatus was used for the initial rate measurements. The changes in absorbance at 310 nm due to the reaction of ferulic acid with PGH synthase and H_2O_2 as a function of time was measured using constant either 21.3 or 25.7 nM enzyme, and varying and high concentration of H_2O_2. Kinetic traces, repeated at least eight times for each set of conditions, were obtained. The initial rates of disappearance of ferulic acid were determined on a time scale of 0.5-1.0 s with experimental errors which were less than 8%.

4.2.3. Oxidation of Ferulic Acid by Arachidonic Acid Catalyzed by PGH Synthase

The rate of ferulic acid oxidation was determined using stopped-flow measurements as described above. One reservoir was used for arachidonic acid solutions, the other for enzyme and ferulic acid, with or without 1 µM hematin, all in 0.1 M phosphate buffer of pH 8.0. The enzyme and ferulic acid mixtures, with or without added hematin, were pre-incubated for 3 min at 30°C. The initial rates of ferulic acid disappearance were determined at 310 nm from kinetic traces that were always linear for times up to 2 s. To avoid the effect of autooxidation of arachidonic acid repetitive traces were obtained within 5 min of preparation of the arachidonic acid solutions. For each reaction mixture placed in the stopped flow apparatus at least seven repetitive traces were obtained, and duplicate or triplicate reaction mixtures were used.

4.2.4. Oxygen Uptake by Arachidonic Acid Catalyzed by PGH Synthase and Stimulated by Ferulic Acid

The rate of arachidonic acid oxidation catalyzed by PGH synthase was determined by monitoring the rate of oxygen consumption as described in Chapter Two. Triplicate experiments were performed in the absence and presence of added hematin. The incubation mixture without added hematin contained 0.1 M phosphate buffer of pH 8.0, 50 nM enzyme and arachidonic acid and ferulic acid in varying concentrations. Cyclooxygenase reactions in the absence of ferulic acid but under otherwise identical conditions were also measured. The concentration of dissolved oxygen was 230 µM at 30°C (10). The rate of arachidonic acid disappearance was determined assuming that 2 mol of oxygen was consumed per mol of arachidonic acid. There is no oxygen uptake by phenolic radicals (4)

4.3. RESULTS

4.3.1. Spectral Behavior of Ferulic Acid in a PGH Synthase Peroxidase Reaction

Spectra of the reaction of ferulic acid with H_2O_2, catalyzed by PGH synthase, were recorded using a Cary 219 spectrophotometer in the 220-380 nm region where ferulic acid absorbs strongly. Maxima are observed at 286 and 310 nm in a phosphate buffer, pH 8.0 (Figure 3.2A, Chapter Three). Measurements at 310 nm were used for the kinetic steady-state experiments because a significant drop in absorbance occurs when ferulic acid is oxidized. Furthermore, there is no significant interference in absorption from H_2O_2 or steady-state concentration of enzyme at 310 nm.

Beer's law was obeyed over the range 5-150 μM ferulic acid in 0.1 M phosphate buffer, pH 8.0, at room temperature, from which we determined ε_{FA} at 310 nm to be 14.84 ± 0.01 $mM^{-1}cm^{-1}$. Reaction of ferulic acid with excess H_2O_2, catalyzed by PGH synthase, was incomplete. Upon addition of more PGH synthase the reaction could be driven to completion. For completed reactions the absorbances of the ferulic acid product at 310 nm was determined for a series of ferulic acid concentrations. The product of ferulic acid oxidation has an $\varepsilon_{FA,\,pr}$ of 7.50 ± 0.15 $mM^{-1}cm^{-1}$. The molar absorptivity coefficient for pure ferulic acid was then corrected for this value and a $\Delta\varepsilon_{FA} = 7.34$ $mM^{-1}cm^{-1}$ was obtained. The latter value was used in the calculations of the initial rate of disappearance of ferulic acid.

Ferulic acid by itself in 0.1 M phosphate buffer, pH 8.0, at 30°C decays spontaneously when exposed to the 310 nm light of the stopped flow apparatus. The overall rates of ferulic acid disappearance obtained in the presence of the enzyme were corrected appropriately using duplicate blank experiments (enzyme, ferulic acid but no arachidonic acid or H_2O_2).

In the presence of excess H_2O_2 and a catalytic amount of PGH synthase, ferulic acid is rapidly oxidized. A single product is formed from ferulic acid as shown by an isosbestic point at 333 nm (Figure 3.2A, Chapter Three). The reaction shown is 85% complete. A subsequent addition of PGH synthase caused an additional drop in absorbance (data not shown). The enzyme and

H_2O_2 were added once more, but did not cause further changes in the product spectrum. Thus the molar absorptivity of the product of ferulic acid oxidation was obtained.

4.3.2. Steady-State Kinetics of the Reaction of Ferulic Acid with H_2O_2, Catalyzed by PGH Synthase.

In order to obtain maximum sensitivity in rate measurements, the reaction conditions were first optimized. From the molar absorptivity measurements at 310 nm, we found that the ferulic acid absorption can be measured in the 5-150 µM range. We also found that a PGH synthase concentration in the 20-50 nM range is optimal to give linear responses in initial rates.

Representative examples of the initial rate measurements are presented in Figure 4.1. The kinetic traces at 310 nm are linear below 1.0 s, and the rates of ferulic acid disappearance were found to be a linear function of $[H_2O_2]$ for five different fixed concentrations of ferulic acid. Applying Beer's law and using $\Delta\varepsilon_{FA}$, the initial rates were calculated from the slopes of the linear parts of the traces, in the 0.5-1.0 s time scale. Data are plotted, for a 1:2 molar ratio of hydrogen peroxide and ferulic acid, as v/E_{tot} versus $[H_2O_2]$ (Figure 4.2). At higher $[H_2O_2]$ the reaction rate increased but the departure from linearity occurred in a shorter time (Figure 4.1).

Our early work (11) demonstrated that the Soret spectrum of the native enzyme, with a maximum at 410 nm, decreases upon reaction with hydroperoxide indicating compound I formation as in other peroxidases. PGH synthase-compound I has reduced absorptivity at 410 nm and an isosbestic point

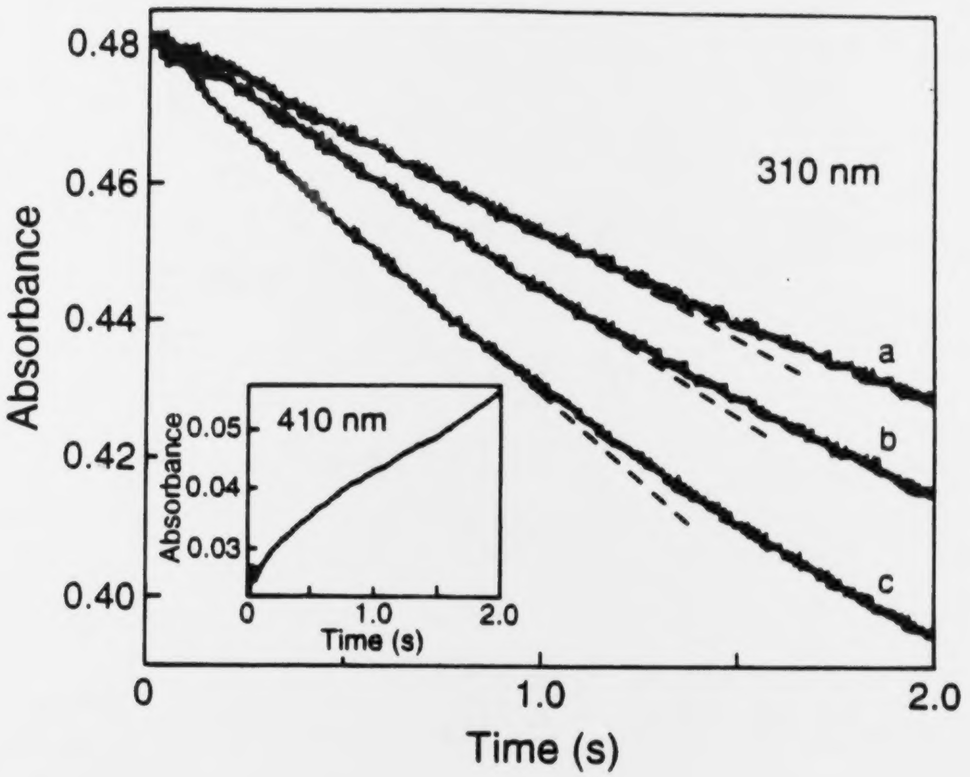

Figure 4. 1: The kinetic traces of FA (32.5 μM) after its reaction with 25.7 nM PGH synthase and 0.365 (a), 0.908 (b) and 1.29 (c) mM H_2O_2 in 0.1 M phosphate buffer, pH 8.0, at 25 ±0.5 °C. *Inset:* Rate of change of absorbance at 410 nm caused by conversion of PGH synthase-compound II back to the native enzyme by its reaction with FA. The reaction mixture contained 32.5 μM FA, 0.5 mM H_2O_2 and 0.15 μM enzyme.

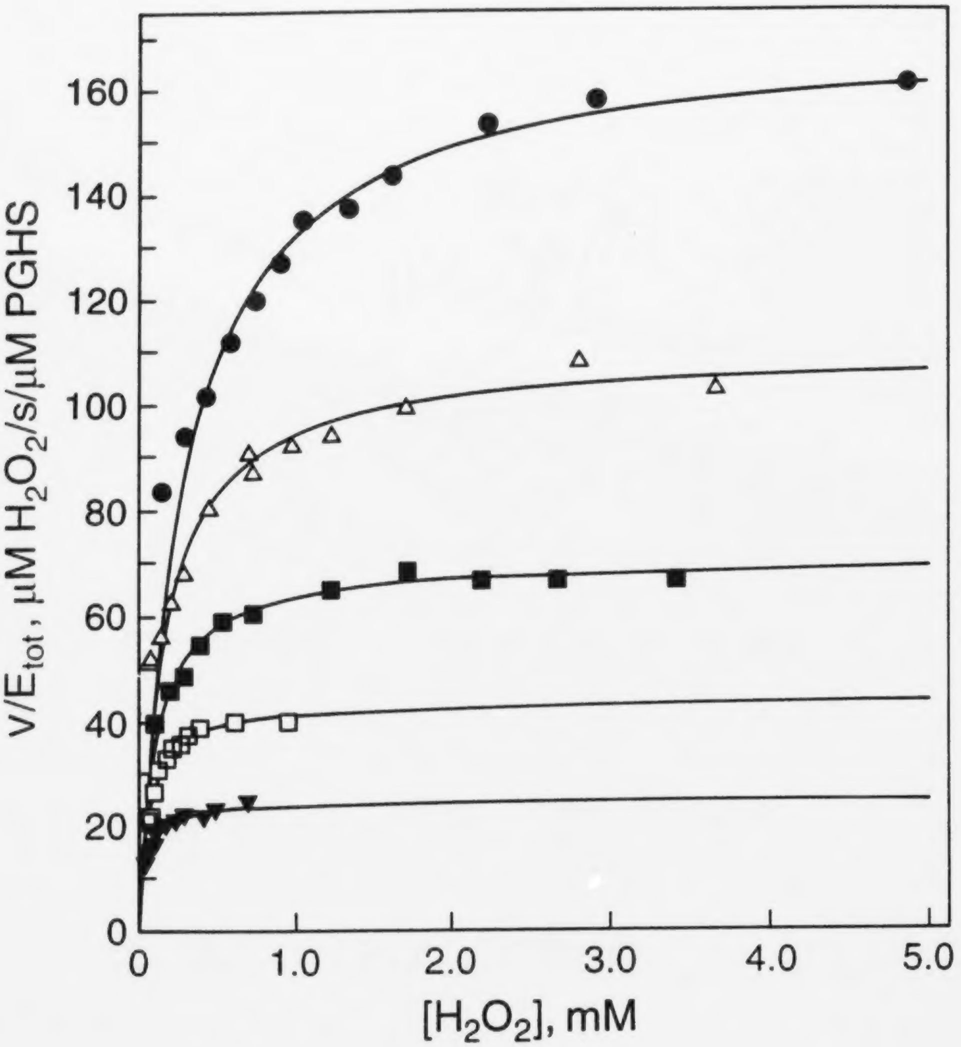

Figure 4. 2. Steady-state initial rates at unit total enzyme concentations of the reduction of the H_2O_2 obtained for the series of [FA]. [FA] are 6.8 (▼), 11.9 (□), 19.8 (■), 31.9 (Δ), and 57.0 (●) µM.; PGH synthase (21.3 nM) having the specific activity of 34.5 µM HAA (mg protein.min)$^{-1}$ is used for these experiments.

with the native enzyme at 426 nm. PGH synthase-compound II has a red-shifted spectrum with a maximum at 420 nm. The only enzyme intermediate observed under our conditions is PGH synthase-compound II. The inset to Figure 4.1 is a kinetic trace at 410 nm (absorption maximum for the native enzyme), showing that PGH synthase-compound II slowly converts to the native enzyme, when 32.5 μM ferulic acid, 0.5 mM H_2O_2 and 0.15 μM enzyme are used. A similar kinetic trace was obtained at 426 nm, the isosbestic point between native enzyme and PGH synthase-compound I. Furthermore, at lower [Ferulic Acid] and higher $[H_2O_2]$, the PGH synthase-compound II conversion is slower (data not shown).

The peroxidase cycle for the oxidation of ferulic acid by PGH synthase and H_2O_2 is as follows:

$$\text{PGH Synthase} + H_2O_2 \xrightarrow{k_1} \text{Compound I} \qquad [4.10]$$

$$\text{Compound I} + \text{FA} \xrightarrow{k_2} \text{Compound II} + \text{FA}\cdot \qquad [4.11]$$

$$\text{Compound II} + \text{FA} \xrightarrow{k_3} \text{PGH Synthase} + \text{FA}\cdot \qquad [4.12]$$

The derivation of the rate law for the reaction of ferulic acid is the same as for *p*-coumaric acid (Appendix A.1, Mechanism I). The rate expression corresponding to eqs. 4.10 - 4.12 is:

$$-d[H_2O_2]/dt = -1/2\ d[FA]/dt = (A\,[H_2O_2]\,[E]_{tot}\,)\,/\,(B + [H_2O_2]) \qquad [4.13]$$

The parameters A and B are :

$$A =(\,k_2k_3\,[FA])\,/\,(k_2 + k_3) \qquad [4.14]$$

$$B = (k_2k_3\,[FA])\,/\,k_1(k_2 + k_3) \qquad [4.15]$$

Consequently,

$$A / B = k_1 \qquad [4.16]$$

The third step, eq. 4.12, is a rate controlling step under our experimental conditions and eq. 4.14 can be simplified to eq. 4.17:

$$A = k_3\,[FA] \qquad [4.17]$$

The experimental values in Figure 4.2 are shown for the initial rates of the oxidation of ferulic acid with PGH synthase and H_2O_2 and the theoretical curve fits to rectangular hyperbolae.

Values for the parameters A and B, (eqs. 4.14 and 4.15) summarized in Table 4.1, were used for linear curve fittings (not shown), applying eqs. 4.16 and 4.17 from above mechanism. From eq. 4.17 the rate constant for the reaction of PGH synthase-compound II with ferulic acid, k_3, is $(2.8 \pm 0.3) \times 10^6\ M^{-1}s^{-1}$. According to eq. 4.16, the linear dependence of the A parameter versus the B parameter (numerical values from Table 4.1) is obtained, with the slope of $k_1 = (6.43 \pm 0.03) \times 10^5\ M^{-1}s^{-1}$ and an intercept not significantly different from zero.

4.3.3. The Stimulation of PGH Synthase Cyclooxygenase Activity by Ferulic Acid

Ferulic acid stimulates the oxidation of arachidonic acid catalyzed by PGH synthase. (Figure 3.1, Chapter Three). A concentration of 0.14 ± 0.02 mM ferulic acid results in 50 % stimulation, when 0.2 mM arachidonic acid and 20 nM enzyme were used. Under the same experimental conditions except for the presence of enzyme, H_2O_2 initiated a non-enzymatic oxidation of arachidonic acid that included oxygen consumption. Ferulic acid efficiently inhibited the non-

Table 4.1. Steady State Kinetic Parameters for the Reaction of Ferulic Acid and H_2O_2 Catalyzed by PGH Synthase[a]

Ferulic acid (μM)	A parameter[b] (s^{-1})	B parameter (mM)
6.8	24.4 ± 0.4[c]	0.035 ± 0.002
11.9	43.5 ± 0.8	0.057 ± 0.003
19.8	69.5 ± 1	0.10 ± 0.01
31.9	109 ± 2	0.16 ± 0.01
57.0	165 ± 3	0.25 ± 0.02

[a] Data were obtained by plotting the initial rate of H_2O_2 disappearance per unit enzyme concentration ($v/[E]_{tot}$) against $[H_2O_2]$ at fixed [FA]. All measurements were performed in 0.1 M phosphate buffer, pH 8.0, at 25 ± 0.5°C.

[b] Parameters A and B were obtained after fitting the rectangular hyperbolae to the experimental data .

[c] Standard deviation.

enzymatic arachidonic acid oxidation, behaving as a classical antioxidant (data not shown).

4.3.4 Cyclooxygenase/Peroxidase Reaction with Equimolar Amounts of Arachidonic and Ferulic Acids

The kinetics of arachidonic acid oxidation (measured with an oxygen electrode) and of ferulic acid oxidation (measured spectrophotometrically on the stopped-flow apparatus) were examined under first order conditions for a molar ratio of arachidonic acid/ferulic acid of 1/1, allowing maximum competition between the two substrates for reaction with the enzyme. The effect of added hematin on both reactions was also examined.

There is a significant rate of oxygen uptake (arachidonic acid disappearance) in the absence of the ferulic acid reducing substrate, and this was determined independently. An example of the magnitude of stimulation of the cyclooxygenase reaction by ferulic acid, i.e., the difference in the initial rates of arachidonic acid disappearance, -dΔ[Arachidonic Acid]/dt, in the presence and absence of ferulic acid, is shown in Figure 4.3A. The corresponding rate of disappearance of ferulic acid is shown in Figure 4.3B, on the same time scale as for the oxygen uptake experiments for direct visual comparison, and on a shorter time scale which was used for quantitative rate measurements.

Stimulation of the cyclooxygenase reaction by ferulic acid was observed for micromolar ratios of arachidonic acid/ferulic acid of 20 /20, 50/50, and 100/100 with and without added hematin. Tables 4.2 and 4.3 show the results along with the corrections for spontaneous decomposition of ferulic acid. Rates of reaction increased with increasing concentrations of substrates and also upon hematin addition, but, remarkably, the ratio of the ferulic acid disappearance to

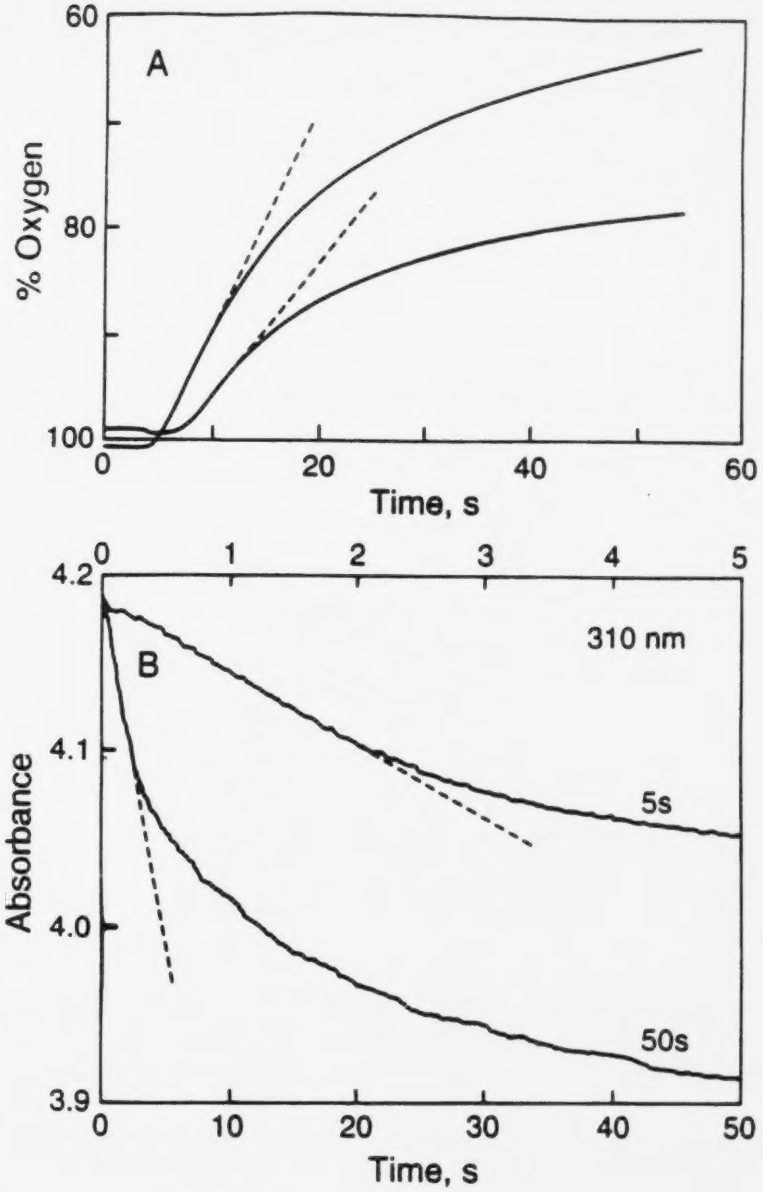

Figure 4.3. Comparison of the PGH synthase-cyclooxygenase **(A)** and peroxidase **(B)** kinetics. **A.** *Upper curve*: Oxygen uptake for 50 μM HAA, 50 μM FA, 35 nM PGH synthase and 1 μM hematin in 0.1 M phosphate buffer, pH 8.0, at 30.0 ± 0.5 °C; *Lower curve:* Same conditions except no FA was added. **B.** kinetic traces at 310 nm of the oxidation of FA for 5 and 50 s. Reaction conditions as in **A** (*Upper curve*).

the stimulated rate of arachidonic acid disappearance remained constant with a value of 2.

$$\frac{-d[\text{Ferulic acid}]/dt}{-d\Delta[\text{Arachidonic acid}]/dt} = \frac{-d[\text{Ferulic acid}]}{-d\Delta[\text{Arachidonic acid}]} = 2 \qquad [4.18]$$

4.3.5 Cyclooxygenase/Peroxidase Reactions with Ferulic Acid in Excess of Arachidonic Acid

Two sets of experiments were performed in this series: (i) arachidonic acid concentration was kept constant and ferulic acid concentration was varied and (ii) ferulic acid concentration was kept constant and arachidonic acid concentration was varied. The first set of experiments was performed in the presence and absence of added hematin; the second set only in the presence of added hematin.

In the first set of experiments the rates of disappearance of the two substrates were monitored for ferulic acid concentrations of 50, 100 and 200 μM and an arachidonic acid concentration of 20 μM ([Ferulic Acid]/[Arachidonic Acid] ratios of 2.5/1, 5/1 and 10/1 in Tables 4.2 and 4.3). The 2-fold increase in ferulic acid concentration from 50 to 100 μM is accompanied by a parallel increase in both the peroxidase reaction and stimulation of the cyclooxygenase reaction (1.5- and 1.4-fold respectively). When the ferulic acid concentration was increased from 100 to 200 μM no change was observed in the rates of both the peroxidase and cyclooxygenase reactions for the holo-/apo- enzyme, whereas inhibition of both activities occurred for the reconstructed enzyme. Maximum rates for both the peroxidase reaction and stimulated cyclooxygenase reaction are attained when the [Ferulic Acid]/[Arachidonic Acid] ratio is 5/1. Even though marked

Table 4.2. Simultaneous oxidation of arachidonic and ferulic acids catalyzed by 40/60 mixture of holo- and apo-PGH synthase: Relation between the rate of ferulic acid (FA) disappearance and the magnitude of its stimulation of arachidonic acid (AA) disappearance[a]

		Stopped-flow measurements[b]			Oxygen uptake measurements[c]			
[FA]/[AA]	μM FA/μM AA	Slope_{tot} (ΔAs^{-1})	Slope_{bl} (ΔAs^{-1})	- d[FA]/dt (μM/s)	Slope_{st} (s^{-1})	Slope_{sp} (s^{-1})	-dΔ[AA/]dt (μM/s)	d[FA]/dΔ[AA]
A. *Same amount of arachidonic and ferulic acids:*								
1/1	20/20	0.0149	0.0090	0.804	0.0041	0.0025	0.368	2.2
1/1	50/50	0.0367	0.0290	1.05	0.0050	0.0030	0.460	2.3
1/1	100/100	0.0510	0.0370	1.91	0.0056	0.0025	0.713	2.7
B. *Arachidonic acid concentration is constant; ferulic acid is in excess:*								
2.5/1	50/20	0.0386	0.0290	1.31	0.0052	0.0025	0.621	2.1
5/1	100/20	0.0520	0.0370	2.04	0.0063	0.0025	0.874	2.3
10/1	200/20	0.0499	0.0360	1.90	0.0067	0.0025	0.966	2.0
C. *Ferulic acid concentration is constant; arachidonic acid is in excess:*								
1/4	20/80	0.0200	0.0150	0.681	0.0033	0.0030	0.069	9.9
1/10	20/200	0.0245	0.0150	1.29	0.0031	0.0031	NS[d]	-
1/2	50/100	0.0392	0.0290	1.40	0.0044	0.0025	0.437	3.2
1/4	50/200	0.0426	0.0290	1.85	0.0033	0.0033	NS	-

[a] 50 nM PGH synthase.

[b] Slope_{tot} represents the initial change in the absorbance at 310 nm for the catalyzed degradation of ferulic acid in the presence of arachidonic acid. Slope_{bl} is the initial slope for the spontaneous decay of ferulic acid; the difference between the two slopes divided by $\Delta\varepsilon_{FA}$ gives the rate of ferulic acid disappearance (-d[FA]/dt in μM/s) for the catalyzed reaction.

[c] Slope_{st} and Slope_{sp} are 1/2 of the initial slopes of the oxygen consumption curves obtained for the catalyzed oxygenation of arachidonic acid in the absence and presence of ferulic acid; the difference between the two slopes multiplied by the amount of dissolved oxygen (230 μM) results in the rates, dΔ[AA]/dt in μM/s.

[d] No stimulation of oxygen uptake by ferulic acid.

Table 4.3. Simultaneous oxidation of arachidonic acid and ferulic acid catalyzed by PGH synthase with added hematin. Relation between the rates of ferulic acid (FA) disappearance and the magnitude of its stimulation of arachidonic acid (AA) disappearance[a]

		Stopped-flow measurements[b]			Oxygen uptake measurements[c]			
[FA]/[AA]	μM FA/μM AA	Slope_{tot} (ΔAs^{-1})	Slope_{bl} (ΔAs^{-1})	- d[FA]/dt (μM/s)	Slope_{st} (s^{-1})	Slope_{sp} (s^{-1})	-dΔ[AA]/dt (μM/s)	d[FA]/dΔ[AA]
A. *Same amount of arachidonic and ferulic acids:*								
1/1	20/20	0.0180	0.0090	1.23	0.0065	0.0040	0.575	2.1
1/1	50/50	0.0400	0.0260	1.91	0.0095	0.0059	0.828	2.3
1/1	100/100	0.0737	0.0348	5.25	0.0135	0.0038	2.23	2.4
B. *Ferulic acid concentration is constant and in excess:*								
20/1	200/10	0.0520	0.0360	2.18	0.0081	0.0035	1.05	2.1
4/1	200/50	0.0620	0.0360	3.54	0.0150	0.0059	2.09	1.7
2/1	200/100	0.0640	0.0360	3.81	0.0140	0.0040	2.30	1.7
C. *Arachidonic acid concentration is constant; ferulic acid is in excess:*								
2.5/1	50/20	0.0527	0.0290	3.23	0.0102	0.0040	1.43	2.3
5/1	100/20	0.0723	0.0370	4.81	0.0124	0.0040	1.93	2.5
10/1	200/20	0.0630	0.0360	3.68	0.0112	0.0040	1.66	2.2
D. *Ferulic acid concentration is constant; arachidonic acid is in excess:*								
1/4	20/80	0.0128	0.0030	1.34	0.0055	0.003	0.575	2.3
1/10	20/200	0.0211	0.0030	2.46	0.0050	0.0025	0.575	4.3

[a] **A**, **C**, and **D:** 35 nM PGH synthase. **B**: 50 nM PGH synthase.
[b] Symbols, units, and corrections made for the spontaneous decay of ferulic acid same as in Table 4.2.
[c] Symbols, units, and calculations same as in Table 4.2.

changes were observed in the rates of reaction as a function of concentration of ferulic acid, the ratio of the rate of ferulic acid disappearance to the stimulated rate of arachidonic acid disappearance remained constant with a value of 2 as in the 1/1 series of the experiments.

In the second set of the experiments, in which arachidonic acid concentration was varied and ferulic acid concentration was kept constant ([Ferulic Acid]/[Arachidonic Acid] ratios of 2/1, 4/1 and 20/1, Table 4.3), the rates of the peroxidase reaction and stimulation of the cyclooxygenase reaction were functions of arachidonic acid concentration but again the ratios of the two rates was constant with a value of 2.

4.3.6 Reaction of PGH Synthase with Arachidonic Acid in Excess of Ferulic Acid

The results obtained with arachidonic acid in excess are considerably different from those described above. There was considerable ferulic acid oxidation but no significant stimulation of arachidonic acid oxidation in all experiments except one (Tables 4.2 and 4.3). The one exception is when 20 µM/80µM ferulic acid/arachidonic acid were reacted with reconstituted enzyme (Table 4.3). The lack of stimulation is more apparent at lower concentration of holo-/apo-enzyme, Table 4.2) than at higher concentrations or reconstructed enzyme, Table 4.3). For 1/4 and 1/10 ratios of ferulic acid to arachidonic acid using PGH synthase as isolated as well as a 1/10 ratio of ferulic acid to arachidonic acid using the reconstructed enzyme, there is no significant stimulation of arachidonic acid disappearance. Consequently, d[Ferulic Acid]/dΔ[Arachidonic Acid] ratios became significantly larger than 2. The results are opposite from what might be anticipated. Since the cyclooxygenase reaction involves arachidonic acid, with excess arachidonic acid a faster rate of its disappearance might be expected yielding a d[Ferulic Acid]/dΔ[Arachidonic Acid] ratio lower than 2. It appears that at higher arachidonic acid concentrations enzyme inactivation takes place.

4.4 DISCUSSION

4.4.1. PGH Synthase, H_2O_2 and Ferulic Acid

Ferulic acid is oxidized with PGH synthase higher oxidation states through two hydrogen atom abstractions yielding free radicals that combine non-enzymatically. Under steady-state conditions, the only enzyme intermediate detected is compound II. The inset to Figure 4.1 shows a kinetic trace for the conversion of PGH synthase-compound II back to the native enzyme. The initial burst in the kinetic traces indicates that yet another intermediate, namely PGH synthase-compound I, is formed in the pre-steady-state phase. The results of the initial rate measurements reveal that ferulic acid is oxidized by PGH synthase higher oxidation state intermediates through the same irreversible ping-pong mechanism as for *p*-coumaric acid (Chapter Two; 12) as is typical for all peroxidases (13).

4.4.2 Arachidonic Acid, Ferulic Acid and H_2O_2

Our experiments show that ferulic acid prevents a non-enzymatic oxidation of arachidonic acid by H_2O_2. In the absence of enzyme, oxygen consumption ceased in the presence of ferulic acid, indicating inhibition of arachidonic acid oxidation. Ferulic acid probably acts as a classical chain-breaking antioxidant by efficiently removing peroxide radicals initially formed from arachidonic acid (14).

4.4.3. Effect of Ferulic Acid on the PGH Synthase-Arachidonic Acid Reaction

In marked contrast to the non-enzymatic reaction, our results show that ferulic acid stimulates the enzyme-catalyzed arachidonic acid oxidation. The concentrations necessary for 50% stimulation of 0.2 mM arachidonic acid oxidation are in the order ferulic acid > *p*-coumaric acid > caffeic acid (Chapter Three) in agreement with their kinetic mechanisms and rate constants for their reactions with PGH synthase compound II. The hyperbolic curves obtained by a plot of O_2 consumption versus [Ferulic Acid] (Chapter Three, Figure 3.1) suggest that ferulic acid is playing an important role in the enzymatic cycle involving arachidonic acid.

4.4.4. Direct Comparison of the Cyclooxygenase and Peroxidase Reactions

In order to acquire a better understanding of what features make a PGH synthase cyclooxygenase reaction similar or different from PGH synthase peroxidase reaction, measurements were made, for identical reaction mixtures consisting of PGH synthase, arachidonic acid and ferulic acid, of the initial rates of disappearance of the substrates. For the cyclooxygenase reaction (reaction of arachidonic acid) the rate of oxygen consumption was measured using an oxygen electrode. For the peroxidase reaction (disappearance of ferulic acid) we utilized the previously developed system of stopped-flow steady-state studies for accurate measurements of initial rates (Chapter Two). The minimum time for measurements on the two instruments are somewhat different but direct comparisons for identical time scales were possible and were made. Free radicals derived from ferulic acid, like those from other monophenols, do not react with molecular oxygen so there is no ambiguity in the measurements.

Furthermore, neither arachidonic acid nor its oxidation products interfere with ferulic acid absorbance at 310 nm.

In the present study the ratio of peroxidase to cyclooxygenase activity was evaluated for series of arachidonic acid and ferulic acid concentrations for two PGH synthase preparations, one using the enzyme as isolated and the other using enzyme reconstructed with added hematin. When the overall rate of arachidonic acid disappearance corrected for the spontaneous rate of arachidonic acid disappearance is related to the corresponding rates of ferulic acid disappearance, constant ratios were demonstrated to exist for a wide variety of experimental conditions (Tables 4.2 and 4.3). The results imply that there is a fixed proportion of the catalytic power of PGH synthase utilized for each of the two substrates, arachidonic acid and ferulic acid.

4.4.5. The Tyrosyl Radical Mechanism

There are two mechanisms in the literature which describe the cycloxygenase and peroxidase reactions. The first is the tyrosyl radical mechanism described in eqs. 4.1 - 4.7. The tyrosyl radical, detected by ESR spectroscopy (15), is formed with a rate constant of 65 s^{-1} (2) by the spontaneous conversion of the conventional Fe^{IV}=O porphyrin π-cation radical form of compound I to an Fe^{IV}=O tyrosyl radical possessing the same number of oxidizing equivalents. Eqs. 4.1-4.5 are based on the *Perspectives in Biochemistry* article (3). We have added eqs. 4.6 and 4.7 since it is necessary when discussing a total mechanism for a free radical chain mechanism to include chain termination steps. Removal of both compounds I and II by competition of phenol with arachidonic acid interferes with the arachidonic acid reaction. Phenolic free radicals do not react with molecular oxygen, but typically react with each other (4) so they act as efficient chain terminators. Thus eqs. 4.1-4.7 lead to a prediction that increasing amounts of the phenol ferulic acid would lead to an ever increasing ratio of ferulic acid/arachidonic acid reacted, which is clearly not the case. It is evident that neither an uncontrolled arachidonic acid reaction nor a rate of arachidonic acid reaction which is faster than that of ferulic acid can occur.

4.4.6. Hydrogen Atom Abstraction from Arachidonic Acid by Compound I followed by Compound II Reduction by Ferulic Acid

The alternative to the tyrosyl radical hypothesis is that a conventional compound I is responsible for hydrogen atom abstraction (Eqs. 4.1, 4.8, 4.4, 4.9 and 4.7 in sequence, listed in the Introduction). These equations predict that no PGG_2 would be detected in the stimulated reaction, in agreement with literature results obtained with ram seminal vesicle microsomes or purified enzyme (16-19). As explained in the original proposal (5), this reaction may consist of abstraction of the 13-*pro-S* hydrogen atom from arachidonic acid by the $Fe^{IV}=O$ group of compound I (a cytochrome P-450-like reaction), followed by proton transfer to a distal basic group of the enzyme and electron transfer to the porphyrin ring, as occurs in the normal peroxidatic mechanism of compound II formation (13). Alternatively, as suggested by a referee, the porphyrin π-cation radical of compound I may play the role we have suggested for the $Fe^{IV}=O$ group. We cannot distinguish between these two possiblities. Our present results confirm the prediction, from the sum of equations 4.1, 4.8, 4.4, 4.9 and 4.7 (Introduction), of a 2:1 ratio of ferulic acid reacted/arachidonic acid reacted; and this excludes the tyrosyl radical chain mechanism. The abstraction of the 13-*pro-S* hydrogen atom from arachidonic acid by a conventional compound I implies that arachidonic acid binds and reacts preferentially with compound I and that ferulic acid reacts preferentially with compound II (5).

PGH synthase reconstituted with manganese porphyrin exhibits considerable cyclooxygenase activity but little peroxidase activity (20-22). Manganese-substituted yeast cytochrome c peroxidase exhibits markedly different properties from the iron holo-enzyme, with about 10% of the normal peroxidatic activity (23). Manganese has different preferred oxidation states from iron. It is not surprising that the manganese-substituted PGH synthase displays different reactivities from the iron enzyme. Its mechanism of action is as yet unknown. PGH synthase mutants also display different ratios of cyclooxygenase to peroxidase activities. Tyr-385 is essential for the cyclooxygenase activity and His-309, His-207 and His-388 are essential for both PHG synthase activities. Their replacement causes notable alterations in the enzyme functions (24, 25). The cause may be alteration of the binding site of arachidonic acid and of the heme environment.

The important conclusion which can be drawn from the present study is that in the presence of reducing substrate an arachidonic acid chain reaction is not possible. At present, the only plausible alternative is a non-chain mechanism in which compound I of PGH synthase, an $Fe^{IV}=O$ porphyrin π-cation radical, is responsible for hydrogen atom abstraction from arachidonic acid.

4.4.7 General Discussion

The tyrosyl radical mechanism and the peroxidase mechanism, as they have been outlined in the literature and in the introduction to this paper, can be distinguished in several ways. The predominant or exclusive product of arachidonic acid oxidation in the presence of phenol is PGH_2 (16-19). This supports the classical peroxidase mechanism in which PGG_2 is consumed stoichiometrically. The measurements of the rates of PGG_2 and PGH_2 formation will be useful in distinguishing between the two mechanisms, but these compounds as well as arachidonic acid do not possess suitable chromophores for stopped-flow spectrophotometric measurements. We will show in the next chapter how a reaction with another fatty acid substrate, cis, cis- eicosa-11,14-dienoic acid, can be exploited for the measurement of the initial rate of formation of the hydroperoxy and hydroxy derivatives of fatty acid and used as a tool in deducing the PGH synthase oxygenase catalytic mechanism. Generally, there is no problem in determination of the amount of phenolic products formed during arachidonic acid oxygenation except that secondary reactions such as polymerization can occur. Ferulic acid forms a single dimer in its horseradish peroxidase reaction (1). In this work have shown that the same product is formed in its PGH synthase reaction. Product analysis studies which include the measurements of the amounts of both arachidonate and phenolate products will be of use in studying the stoichiometry of the reaction but these remain to be done. In this study we established the stoichiometry between the amount of arachidonic acid and the phenol ferulic acid reacted by using the kinetic method of initial rate measurements under steady state conditions, by which the effects of secondary reactions and enzyme inactivation are minimized. Our kinetic experiments showed that there is a constant 2:1 stoichiometry between the amount of the phenol ferulic acid and arachidonic acid reacted. This result contradicts the tyrosyl radical mechanism as outlined in eqs. 4.1-4.7.

There is an alternative possibility which deserves consideration. The tyrosyl residue is a phenol, and thus it appears reasonable that it may participate in some fashion, although not in an independent free radical chain reaction. The following pair of reactions are a possibility:

$$\text{Compound II} + \cdot\text{PGG}_2 \rightarrow \text{Compound-I'-Tyr}\cdot + \text{PGG}_2 \qquad [4.5]$$

$$\text{Cpd-I'-Tyr}\cdot + \text{HOPh} \rightarrow \text{Compound II} + \text{OPh} \qquad [4.19]$$

$$\mathrm{HOPh} + \cdot\mathrm{PGG_2} \rightarrow \mathrm{OPh} + \mathrm{PGG_2} \qquad [4.9]$$

Thus the sum of eqs. 4.5 and 4.19 is eq. 4.9, part of the peroxidase mechanism. It is not possible at this time to distinguish between the two-step and one-step processes; they could both be in operation. Evidence which supports the two-step hypothesis is that tyrosyl radicals were not detected in the presence of phenol (20).

In conclusion, our results for reaction mixtures of arachidonic acid and a phenol preclude an independent tyrosyl radical chain mechanism, but they do not preclude participation of the tyrosyl residue and radical in a different fashion. A classical peroxidase cycle, in which arachidonic acid is a preferred compound I substrate and a phenol such as ferulic acid reduces compound II, can account for all of the results.

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CHAPTER FIVE

OXYGENATION REACTIONS OF PROSTAGLANDIN ENDOPEROXIDE SYNTHASE AND SOYBEAN LIPOXYGENASE. THE FORMATION OF HYDROPEROXY AND HYDROXY DERIVATIVES OF cis, cis-EICOSA-11, 14-DIENOIC ACID IN THE PRESENCE AND ABSENCE OF TROLOX C*

5.1. INTRODUCTION

Prostaglandin endoperoxide synthase (PGH synthase)** is capable of oxidizing di- or polyunsaturated fatty acids incorporating a single molecule of oxygen in a reaction that otherwise resembles the formation of prostaglandins from 20:4 (1). As in 20:4 oxidation, the hydrogen atom abstraction and oxygen addition occur at a suitably located 1,4-cis, cis pentadiene system of the fatty acid but results in the formation of a hydroperoxy- and/or hydroxy-trans, cis conjugated fatty acid product. During the PGH synthase catalyzed oxidation of cis,cis-eicosa-11,14-dienoic acid (20:2), 11-OOH-20:2 and 11-OH-20:2 derivatives can be produced in the following reaction sequence (2):

$$\text{11,14-cis, cis-20:2} + O_2 \xrightarrow{\text{oxygenase}} \text{11-OOH-12-trans, 14-cis -20:2} \quad [5.1]$$

$$\text{11-OOH-12-trans, 14-cis -20:2} \xrightarrow{\text{peroxidase}} \text{11-OH-12-trans, 14-cis -20:2} \quad [5.2]$$

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** Abbreviations: PGH synthase, prostaglandin endoperoxide synthase; 20:4, arachidonic acid; 20:2, eicosa-11, 14-dienoic acid; 11-OOH-20:2, 11-hydroperoxy eicosa-12, 14- dienoic acid; 11-OH-20:2, 11-hydroxy eicosa-12, 14-dienoic acid; 15-OOH-20:2, 15-hydroperoxy eicosa-11, 13-dienoic acid; 15-OH-20:2, 15-hydroxy eicosa-11, 13-dienoic acid; DDC, diethyldithiocarbamate; TMPD, N, N, N', N'-tetramethyl-*p*-phenylenediamine; Trolox C, 6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid; ε, extinction coefficient.

Although the products from the above PGH synthase catalyzed reaction have no structural similarity to prostaglandins, the 20:2 and 20:4 oxygenation reactions have many features in common (1). Both substrates exhibit similar affinity for the enzyme, a requirement for peroxide to initiate the reaction, and a turnover-mediated inactivation of the enzyme.

During the lipoxygenase catalyzed oxygenation of 20:2 a single molecule of oxygen is added at carbon 15 and a hydroperoxide derivative, 15-OOH-20:2, is formed (1, 3). This labile hydroperoxide can be reduced chemically to the more stable 15-OH-20:2 derivative. After similar reductive derivatization of the oxygenation products of 20:2 from the PGH synthase catalyzed oxidation (1), another isomer, 11-OH-20:2 (shown in eq. 5.2) was isolated. Thus the oxygenation of 20:2 by soybean lipoxygenase and PGH synthase have in common the addition of single molecule of oxygen but they have different stereospecificities.

It appeared to be extremely difficult to study the reaction mechanism of PGH synthase-cyclooxygenase by kinetic methods. The quantitative conversion rates of the substrate 20:4 are determined exclusively from the oxygen consumption measurements. 20:4 and prostaglandin are spectroscopically silent species and no fast technique such as stopped flow spectrophotometry is of use for the initial rate determinations. The overall stoichiometry of the cyclooxygenase reaction was determined from the amount of prostaglandin formed by radioisotopic and chromatographic methods on time scales far different from the oxygen consumption measurements (4-7). Therefore, no direct comparisons of the rates of oxygen consumption and the rates of prostaglandin formation are possible; it is unknown whether the kinetic methods would provide similar or different results from the product analysis methods.

20:2 is a suitable substrate for the application of the kinetic method in the investigation of the reaction mechanism of PGH synthase. Here we developed a stopped flow method for the measurements of the initial rates of formation of the oxygenation products of 20:2 in the PGH synthase catalyzed reaction. The method relies upon the fact (3) that the major products of 20:2 absorb at 235 nm due to the formation of a conjugated system of double bonds (eqs. 5.1 and 5.2). The stopped flow method is directly comparable with the polarographic method of the oxygen consumption measurements. This provides for the first time two comparable ways of studying the initial phase of the oxygenation reaction of PGH synthase, where the secondary reactions, the autooxidation of substrate and the inhibition of the enzyme are significantly reduced.

Using the combination of the above two methods we will demonstrate that the PGH synthase oxygenase reaction operates through a different catalytic mechanism from the soybean lipoxygenase oxygenase reaction. The overall mechanism and stoichiometry between oxygen consumed and hydroperoxide formed in the lipoxygenase catalyzed reaction are very well established (8). Here we propose a mechanism for the PGH synthase oxygenation reaction of 20:2 which includes both one- and two-electron transfer processes and validate the previous conclusion (Chapter Four) that the two enzyme activities of PGH synthase, as an oxygenase and a peroxidase, are intimately connected.

5.2. MATERIALS AND METHODS

5.2.1. Materials

20:2 and 20:4 were obtained from Sigma. The fatty acids were dissolved in deaerated absolute ethanol and stored in an inert atmosphere at -70^{o}C. Trolox C was obtained from Aldrich. The stock solution of Trolox C was in 7 % ethanol and it was prepared just before use. The amount of ethanol in the final reaction mixtures had no effect on the rates of the oxygenase reaction.

Phenol was supplied by British Drug House. A stock solution of phenol was prepared fresh prior to use in deionized water. Hematin from bovine blood and the sodium salt of DDC were both from Sigma and of the highest purity available. All reagents were kept on ice and protected from light.

Commercial soybean lipoxygenase (EC 1. 13. 1. 13) was purchased from Sigma. As recommended by the manufacturer, soybean lipoxygenase specific activity was assayed by following the production of linoleic acid hydroperoxide at 234 nm, and a value of 250 units/mg was obtained.

PGH synthase (EC 1. 14. 99. 1) was isolated and purified from ram seminal vesicle microsomes using a procedure as described (Chapter Two). The purified enzyme obtained was 34 % holoenzyme. It had a specific cyclooxygenase activity of 44 μM 20:4 min^{-1} mg^{-1} when the reaction mixture contained 50 μM 20:4, 50 nM enzyme, 1 μM hematin and 1 mM phenol in 0.1 M phosphate buffer, pH 8.0, at 30°C.

5.2.2. Spectral Measurements

Spectra were recorded on a Beckman DU-650 spectrophotometer using 1-cm cuvettes placed in a temperature-controlled holder. For spectral measurements PGH synthase (35 nM enzyme and 1 μM hematin) or soybean lipoxygenase (5 nM) were dissolved in 0.1M phosphate buffer, pH 8.0, and the reaction was initiated with 70 μM of 20:2 at 25°C. Spectra were recorded for the 220-500 nm region. The intervals between scans were 30 s and the scan speed was 2400 nm/min. The reference spectrum was that of PGH synthase or soybean lipoxygenase in the buffer solution. The UV spectra of these solutions exibited an absorbance maximum at 235 nm which has been attributed to the oxidation of 20:2 to form conjugated diene products in both the lipoxygenase and PGH synthase reactions (3, 9).

5.2.3. Soybean Lipoxygenase Reaction with Eicosadienoic Acid

The oxidation of 20:2 catalyzed by soybean lipoxygenase was followed in two different ways: by measuring oxygen consumption with a Yellow Spring Instrument model 53 oxygen monitor, and spectrophotometrically, by measuring the hydroperoxide production at 235 nm with a Photal stopped-flow spectrophotometer, RA-601. Two assays were performed under identical conditions, using different amounts of 20:2 (10-200 µM) and 45.5 nM soybean lipoxygenase in 0.1M phosphate buffer, pH 8.0, at 30°C. The concentration of soybean lipoxygenase was calculated from the absorbance at 280 nm using an $\varepsilon = 1.6 \times 10^5\ M^{-1}cm^{-1}$ (10).

The initial rates, µM/s, were calculated from the maximum slopes of the oxygen consumption curve and the hydroperoxide formation curve using the concentration of dissolved oxygen (230 µM, ref. 11) and the molar absorptivity of 15-OOH-20:2 at 235 nm ($\varepsilon = 23\ 292\ M^{-1}cm^{-1}$, ref. 3). The initial rates of oxygen consumption, $-d[O_2]/dt$, and hydroperoxide formation, $d[ROOH]/dt$, were compared for identical amounts of substrate 20:2 in the two reaction mixtures.

A comparison was also made between the rates of oxygen consumption, monitored by the oxygen electrode, and the rates of hydroperoxide formation, monitored with a Beckman DU-650 spectrophotometer. The experimental conditions were the same as above except that the soybean lipoxygenase concentration was ten times lower (4.5 µM) to slow down the reaction and to allow accurate measurements of the initial rates with the conventional spectrophotometer. Oxygen consumption rates were measured for the same mixtures and the data obtained by the two methods were compared.

5.2.4. PGH Synthase-Oxygenase Reaction with Eicosadienoic Acid

The oxidation of 20:2 catalyzed by PGH synthase was followed in a manner similar to the soybean lipoxygenase reaction. The rate of oxygen consumption was measured with the oxygen electrode and the rate of product formation at 235.0 nm with the stopped-flow apparatus. The absorbance change at 235.0 nm is specific for the electronic transitions from conjugated double bonds of fatty acid derivatives and cannot distinguish between hydroperoxide and hydroxide products having identical conjugated diene parts of the molecule (3). Based on the results of Graff et al. (3), Odenwaller et al. (9) used the extinction coefficient of 24 x10^4 $M^{-1}cm^{-1}$ in their study with 20:2 and manganese-PGH synthase. We used the same value in our calculations.

The enzyme was preincubated in buffer containing hematin at 30^oC for 3 min. For a single experiment, 20:2 was added through the side of the electrode holder immediately before oxygen consumption measurements which were typically completed in less than one min; 20:2 was kept in the reservoir of the stopped flow apparatus no longer than 3 min, long enough to complete 8-10 measurements. The reaction mixtures contained various concentrations of 20:2 (10-300 μM), 35 nM enzyme and 1 μM hematin in 0.1 M phosphate buffer, pH 8.0, at 30^oC. Blank experiments showed that 20:2 was not autooxidized significantly on the time scale of the initial rate measurements in both the oxygen electrode and stopped flow experiments.

5.2.5. Effect of Phenol and DDC on the PGH Synthase Catalyzed Oxygenation of Eicosadienoic acid

Stimulatory and/or inhibitory effects of phenol and DDC on 20:2 oxygenation was followed by oxygen electrode at fixed amount of 20:2 (50 μM), 35 nM enzyme, 1 μM hematin, and varied amounts of phenol or DDC, in 0.1 M phosphate buffer, pH 8.0, at 30°C. The obtained initial rates of oxygen consumption were plotted as a function of [phenol] or [DDC] and corresponding maximal rates and K_m values were compared.

5.2.6. PGH Synthase-Peroxidase Reaction with Trolox C

The peroxidase assay was performed using the Beckman DU-650 spectrophotometer. Trolox C (100 μM) was added to a reconstituted enzyme (10 nM enzyme and 0.5 μM hematin) dissolved in 0.1 M phosphate buffer, pH 8.0, at 25°C and the reaction was initiated with hydrogen peroxide (250 μM). Trolox C has an absorption peak at 288.0 nm and its oxidation product at 269.0 nm. There are two isosbestic points between Trolox C and its peroxidatic product, at 234.5 and 286.0 nm. Of interest to us was the isosbestic point at 234.5 nm. This value is essentially the same as the value for the maximum of the conjugated diene (235.0 nm) formed during the oxidation of 20:2. This allowed monitoring the rate of formation of hydroperoxide and/or hydroxide of 20:2 in the presence of Trolox C. Screening tests with other peroxidase reducing substrates, such as phenol, DDC, ascorbate and TMPD, showed that there are no suitable isosbestic points in their reaction spectra so that they would interfere with the conjugated diene absorption that we wanted to measure. This makes Trolox C one of the rare reducing substrates appropriate for similar type of experiments.

5.2.7. Effect of Trolox C on PGH Synthase-Oxygenase Reaction

The final set of experiments was conducted with a fixed amount of 20:2 (50 μM), different concentrations of Trolox C (10-300 μM), 35 nM enzyme, 1 μM hematin in 0.1 M phosphate buffer, pH 8.0, at 30°C. The rate of oxygen consumption was measured with the oxygen electrode and the rate of product formation at 235.0 nm with the stopped-flow apparatus. Data were plotted as a function of Trolox C concentration and maximal rates and K_m values were compared with the values obtained with phenol or DDC. The initial rates (μM/s) obtained for the same concentrations of Trolox C using the stopped flow and oxygen electrode measurements were also compared.

5.3. RESULTS

5.3.1. Optical Changes Associated with Oxygenation of Eicosadienoic Acid.

The spectral changes observed in the oxygenation of 20:2 were similar in the soybean lipoxygenase and PGH synthase reactions. Figure 5.1 shows the reaction catalyzed with PGH synthase only. The progress of the reaction is accompanied with a large increase in absorbance in the 220-346 nm region with a broad maximum at 235.0 nm. A weak absorbance maximum appeared later at 280.0 nm due to a nonenzymatic degradation of the oxidized products, as previously noticed (9). An extensive loss of the enzyme heme prosthetic group was concomitant with the progress of the PGH synthase-oxygenase reaction. The difference spectra in the Soret region (inset to Figure 5.1) indicated that all heme initially present was completely lost after 2.3 min. Although not shown, the absorbance of the conjugated diene products continued to increase even after 2 min, indicating that some non-heme enzyme reaction, probably involving free-radicals occurred.

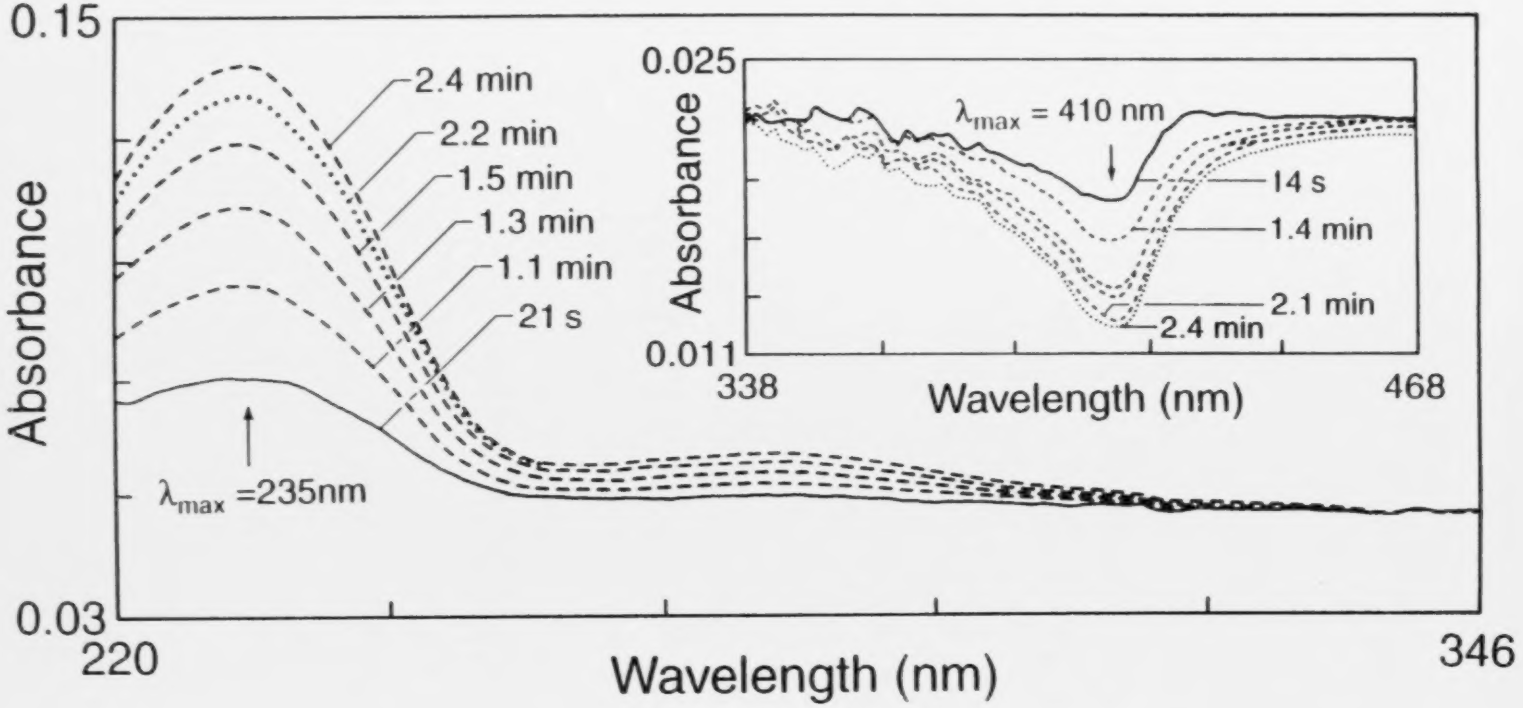


Figure 5.1. Difference absorption spectra showing the formation of conjugated diene products during the PGH synthase (35 nM, 1 μM hematin) catalyzed oxygenation of 20:2 (70 μM). *Inset:* Difference absorption spectra observed in Soret region during the same reaction.

5.3.2. Optical Evidence for the Specific Role of Hydroperoxide in the Initiation of Enzymatic Activity

Figures. 5.2 and 5.3 demonstrate the time courses of product formation at 235.0 nm for the oxidation of 20:2 catalyzed by PGH synthase and soybean lipoxygenase. At the lowest concentration of 20:2 the kinetic traces have a similar biphasic behavior in both systems. An initial drop in absorbance is observed in the pre-steady state phase and there is a sigmoidal increase in absorbance in the steady state phase. The pre-steady state drop in absorbance was not detectable in the kinetic traces obtained at higher 20:2 or enzyme concentrations.

5.3.3. Oxygen Consumption Measurements

After a lag period in the initiation phase a single sigmoidal curve for the steady state phase is observable in the oxygen consumption traces in both PGH synthase and soybean lipoxygenase catalyzed oxygenations of 20:2. The inset to Figure 5.3 shows the oxygen consumption trace obtained in a reaction catalyzed by soybean lipoxygenase and the inset to Figure 5.7 in a reaction catalyzed by PGH synthase in the presence of Trolox C. The oxygen consumption trace of 20:2 in the absence of Trolox C has a similar shape to that in its presence but the overall reaction is slower (data not shown).

5.3.4. Kinetics and Stoichiometry of the Soybean Lipoxygenase Reaction

The results obtained from reactions of several different concentrations of 20:2 with two different concentrations of soybean lipoxygenase were similar in form to those shown in Figure 5.3.

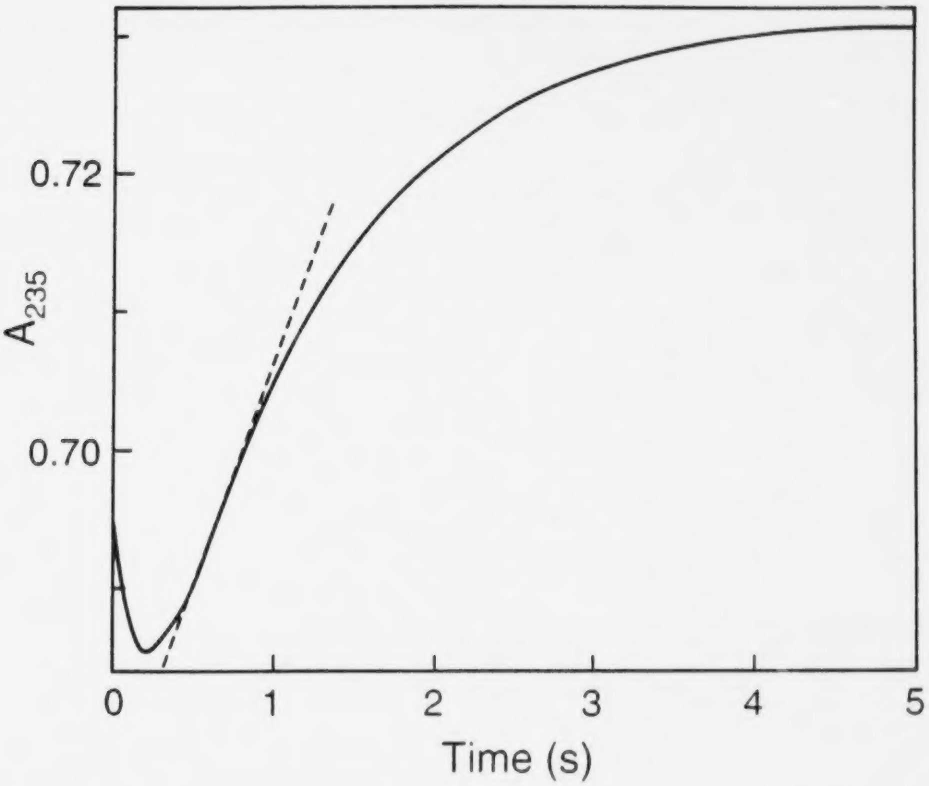


Figure 5.2. Stopped-flow kinetic trace of the reaction between 20:2 and oxygen catalyzed by PGH synthase. Relative absorbance change was followed at 235 nm. Conditions: 125 µM 20:2 , 30 nM PGH synthase, 1 µM hematin in 0.1 M phosphate buffer, pH 8.0, at 30ºC.

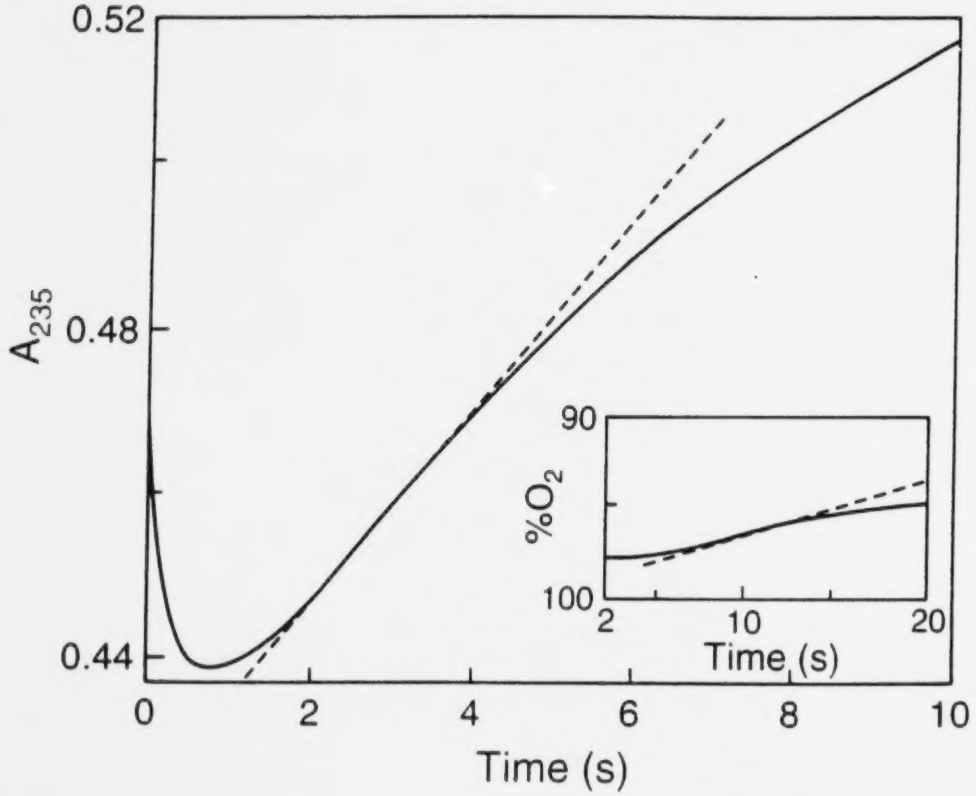


Figure 5.3. Stopped flow kinetic trace at 235 nm indicating the formation of conjugated diene hydroperoxide from 20:2 catalyzed by soybean lipoxygenase. Conditions: 10 μM 20:2 and 45.4 nM soybean lipoxygenase in 0.1 M phosphate buffer, pH 8.0, and at 30°C. *Inset:* Oxygen uptake trace during the same reaction.

The initial rates of hydroperoxide formation, d[ROOH]/dt, versus the initial rates of oxygen consumption, $-d[O_2]/dt$, for a soybean lipoxygenase concentration of 4.5 nM are compared in Figure 5.4A. The slope of 0.92 indicates that within experimental error the following relation is valid:

$$d[ROOH] : -d[O_2] = 1:1 \qquad [5.3]$$

The small positive intercept may be indicative of infinitesimal oxygen consumption in a soybean lipoxygenase autooxidation reaction (12), but no correction to $-d[O_2]/dt$ is applied in Figure 5.4A.

For a 10-fold increase in enzyme concentration (45.5 nM) the results in Figure 5.4B are obtained. In this plot the $-d[O_2]/dt$ values are corrected by 25% for the enzyme inactivation by oxygen, as recommended by Kemal et al. (12). Again a linear plot is obtained with a slope of 1 within experimental error.

5.3.5. Kinetics and Stoichiometry of PGH Synthase Reaction

The rate of the product formation is not identical to the rate of the oxygen consumption in the PGH synthase-oxygenase reaction of 20:2 (Figure 5.5). Both curves showed a saturation behaviour with [20:2]. When we compared the rates for identical [20:2], the values obtained by stopped flow spectrophotometry were always two times higher than the values obtained by the oxygen electrode (Table 5.1). The relationship between the two rates leads to the stioichiometry of conjugated diene product formed and oxygen consumed as follows:

$$d[\text{conjugated diene}] : -d[O_2] = 2 : 1 \qquad [5.4]$$

Since classical peroxidase electron-donors were not supplied to the assay mixtures the above relation applies to a spontaneous oxygenase reaction. The concentration of the stabilizing agent, DDC, was kept constant at 0.65 µM. As a reducing substrate, DDC will be consumed faster than oxygen and will disappear from the solution during the early stage (before 1s) of the reaction (13). As

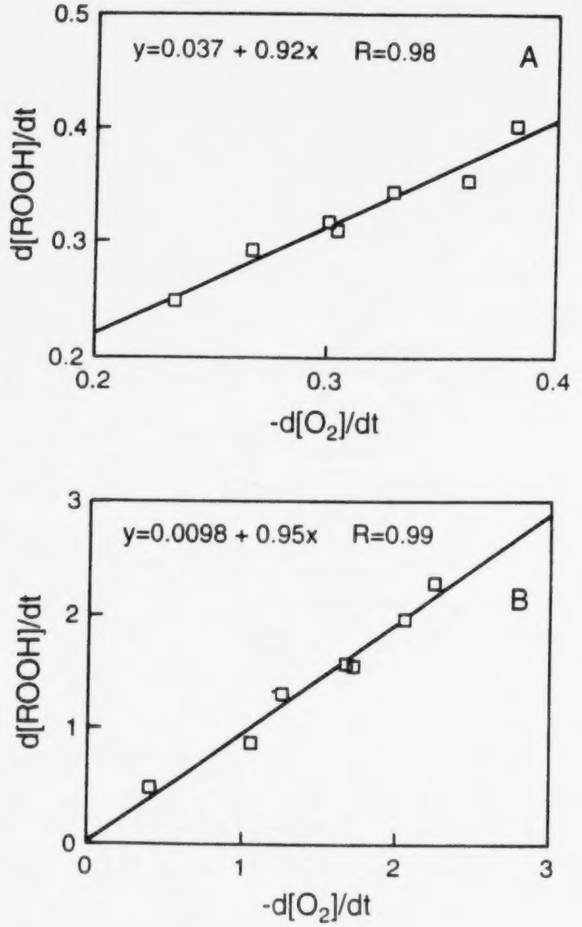


Figure 5.4. Plots of the initial rate of hydroperoxide formation, d[ROOH]/dt, and the initial rate of oxygen consumption, $-d[O_2]/dt$, during the soybean lipoxygenase catalyzed oxygenation of 20:2 . Both rates are in μM/s and the points are obtained for the same [20:2]. The measurements were made for (A) 4.5 nM and for (B) 45.5 nM soybean lipoxygenase and 10-200 μM 20:2. d[ROOH]/dt were measured by (A) conventional and (B) stopped-flow spectrophotometry and $-d[O_2]/dt$ by use of an oxygen electrode.

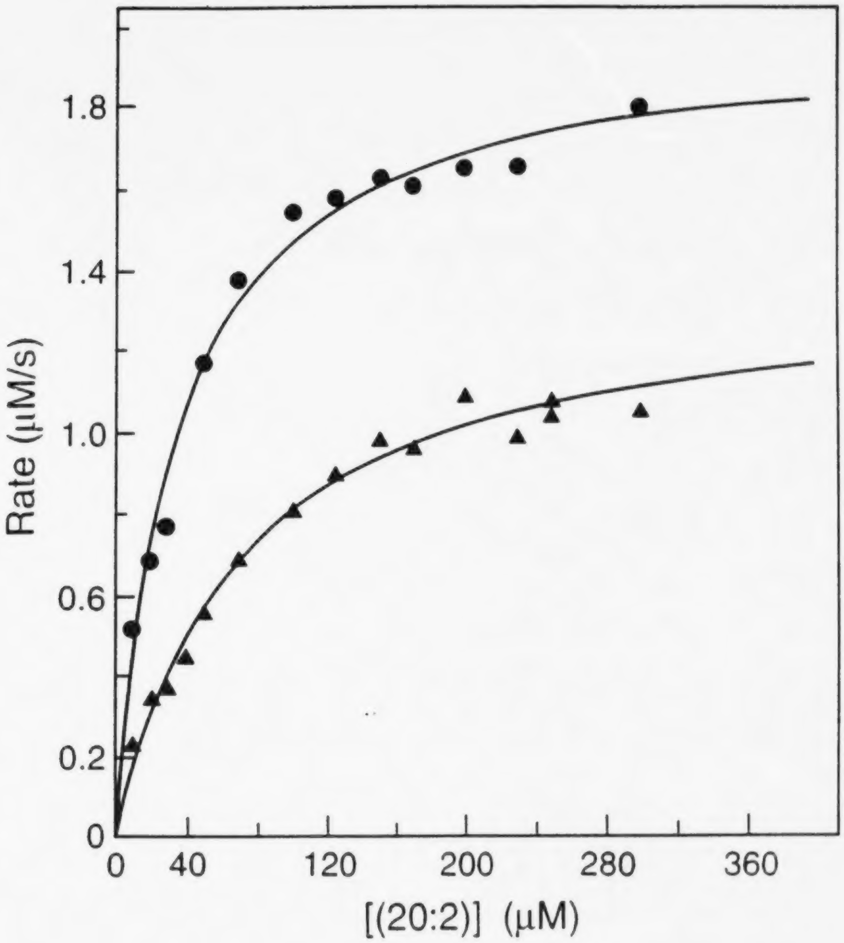


Figure 5.5. Saturation curves showing that the rates of oxygen consumption and the rates of conjugated diene formation during the PGH synthase catalyzed oxygenation of 20:2 are not equal. (▲) the rate of oxygen consumption (●) the rate of products formation at 235 nm versus [20:2] in its reaction with 35 nM PGH synthase and 1 μM hematin.

Table 5.1. Relationship between the rates of conjugated diene formation and oxygen consumption during the oxygenation of 20:2 by PGH synthase in the presence and absence of Trolox C [a]

Substrate, µM		Initial rate, µM/s [b]		
20:2	Trolox C	d[Conjugated diene]/dt	$-d[O_2]/dt$	d[Conjugated diene]:-$d[O_2]$
A. *Spontaneous oxygenase reaction*				
10		0.531	0.230	2.3
30		0.769	0.371	2.1
50		1.17	0.561	2.1
150		1.63	0.979	1.7
300		1.99	1.05	1.9
B. *Oxygenase reaction stimulated with Trolox C*				
50	10	1.08	0.537	2.0
50	50	1.71	0.821	2.1
50	70	1.72	0.851	2.0
50	90	1.89	0.862	2.2
C. *Oxygenase reaction inhibited with Trolox C*				
50	150	1.85	0.805	2.3
50	175	1.56	0.714	2.2
50	200	1.41	0.657	2.1
50	250	1.10	0.438	2.5

[a] 35.5 nM PGH synthase and 1 µM hematin in 0.1 M phosphate buffer, pH 8.0, at 30°C. [b] Formation of conjugated diene was followed by stopped-flow spectrophotometry and oxygen consumption by use of an oxygen electrode.

shown in Figure 5.1 the PGH synthase-oxygenase oxidation of 20:2 continued to occur well after 20 s.

5.3.6. Oxidation of Trolox C with H_2O_2 and PGH Synthase

The change in ratio, if any, between the rate of oxygen consumption and conjugated diene formation that might occur in the presence of some peroxidase electron donors could hardly be predicted from the present knowledge of the PGH synthase reaction mechanism. To get more information we studied the peroxidase reaction separately from the oxygenase reaction, using H_2O_2 peroxide and Trolox C as substrates. Characteristic changes in optical spectra during the oxidation of Trolox C are shown in Figure 5.6. The Trolox C maximum absorbance at 288.0 nm decreased and a new absorbance maximum at 269.0 nm appeared. The presence of two isosbestic points at 234.5 and 268.0 nm indicates that a single product was formed. The identical spectral changes were obtained in a horseradish peroxidase catalyzed reaction (data not shown). The product exhibits additional absorption maxima at 418.5 nm and yet another isosbestic point with Trolox C at 398.5 nm (this part of the spectra was not shown). No oxygen consumption during the reaction was obtained, indicating that molecular oxygen is not involved in PGH synthase peroxidase-mediated oxidation of Trolox C.

5.3.7. Effect of Trolox C on Eicosadienoic Acid Oxygenation Catalyzed by PGH Synthase

To elucidate the effects of electron donors such as Trolox C on the rate of conjugated diene formation we used the fact that there was no significant change in absorbance for Trolox C and its product at 235.0 nm so that any increase in absorbance at this wavelength is attributable only to the product formation from

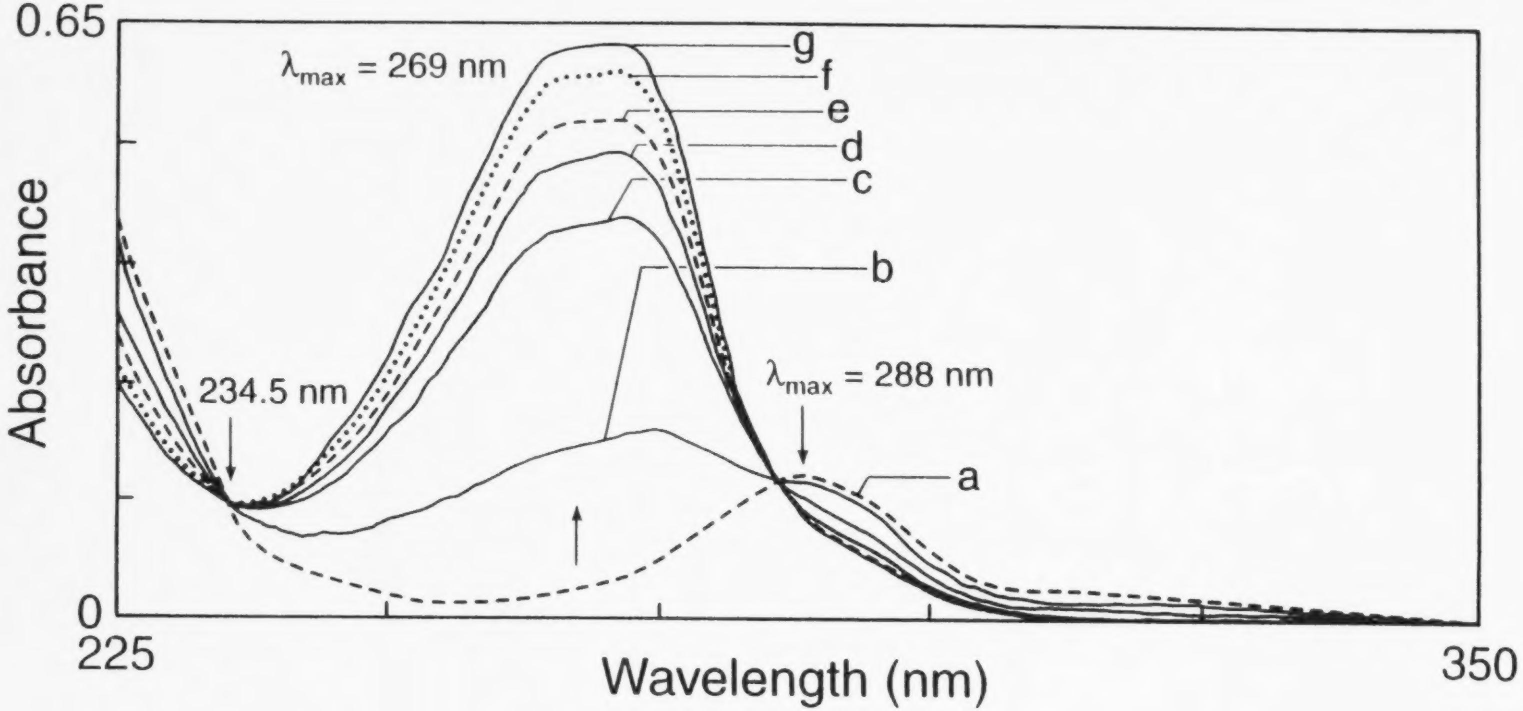


Figure 5.6. Spectral changes of PGH synthase catalyzed oxidation of Trolox C with H_2O_2. The spectra were recorded (a) 0, (b) 0.5, (c) 1.3, (d) 2.3, (e) 3.3, (f) 6.3, and (g) 9.3 min after mixing 100 μM Trolox C, 250 μM H_2O_2, 10 nM PGH synthase and 0.5 μM hematin.

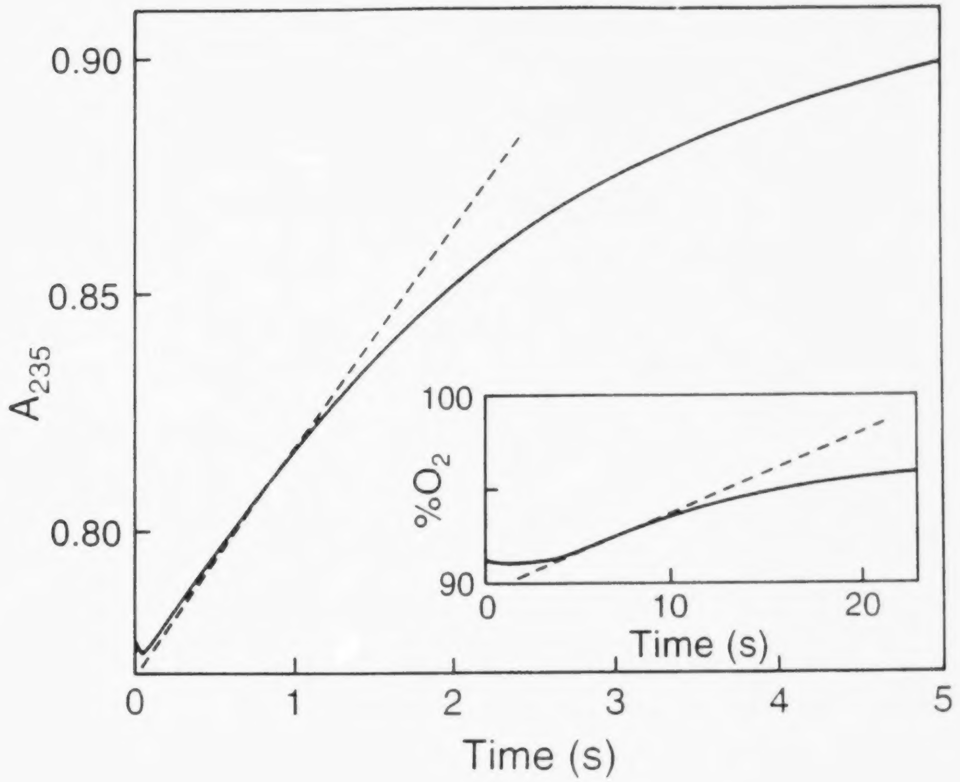


Figure 5.7. Comparison of progress curves of the product formation and oxygen consumption in a 20:2 and PGH synthase-oxygenase reaction stimulated with Trolox C. Stopped-flow kinetic trace obtained at 235 nm. Conditions: 50 μM 20:2, 90 μM Trolox C, 35 nM PGH synthase and 1 μM hematin in 0.1 M phosphate buffer, pH 8.0, at 30°C. *Inset:* Oxygen consumption time course obtained under identical conditions.

20:2 . Figure 5.7 shows a typical stopped-flow kinetic trace recorded in the presence of Trolox C. The curve shows a small initial (100 ms) decrease in absorbance (hydroperoxide consumption in the initiation phase) followed by an increase within the next 5 s (hydroperoxide/hydroxide formation during the steady-state phase). Except for the reaction being faster, no substantial difference from the reaction in the absence of Trolox C was obtained. The inset to Figure 5.7 shows the trend in the oxygen consumption under the same conditions.

The kinetics of Trolox C dependent oxidation of 20:2 was studied at constant [20:2] and for a series of [Trolox C]. Results are summarized in Figure 5.8 and Tables 5.1 and 5.2. Trolox C modulates 20:2 oxidation by (1) stimulating the reaction at lower concentrations until a saturation occurred at approximately 100 μM Trolox C, and (2) inhibiting the reaction at higher concentrations (more than 100 μM Trolox C) (Figure 5.8). The ratios of the initial rates of the conjugated diene formation and oxygen consumption in the presence of Trolox C are listed in Table 5.1. As in case of the spontaneous oxygenase in the stimulated oxygenase the ratio between the rate of fatty acid product formation and the rate of oxygen consumption is 2:1. If the concentration of Trolox C was increased, both rates increased by the same amount so that the ratio remained unchanged. Surprisingly, inhibition by higher concentrations of Trolox C did not affect the 2:1 ratio (Table 5.1).

5.3.8. Comparison of Trolox C with Other Peroxidase Electron Donors

In agreement with the results shown in Figure 5.8 and Table 5.1, results in Table 5.2 show that Trolox C increases the maximum rate of the oxygen consumption about 2-fold in comparison to the spontaneous reaction under standard assay conditions i.e., 50 μM 20:2 , 35.5 nM PGH synthase, and 1 μM

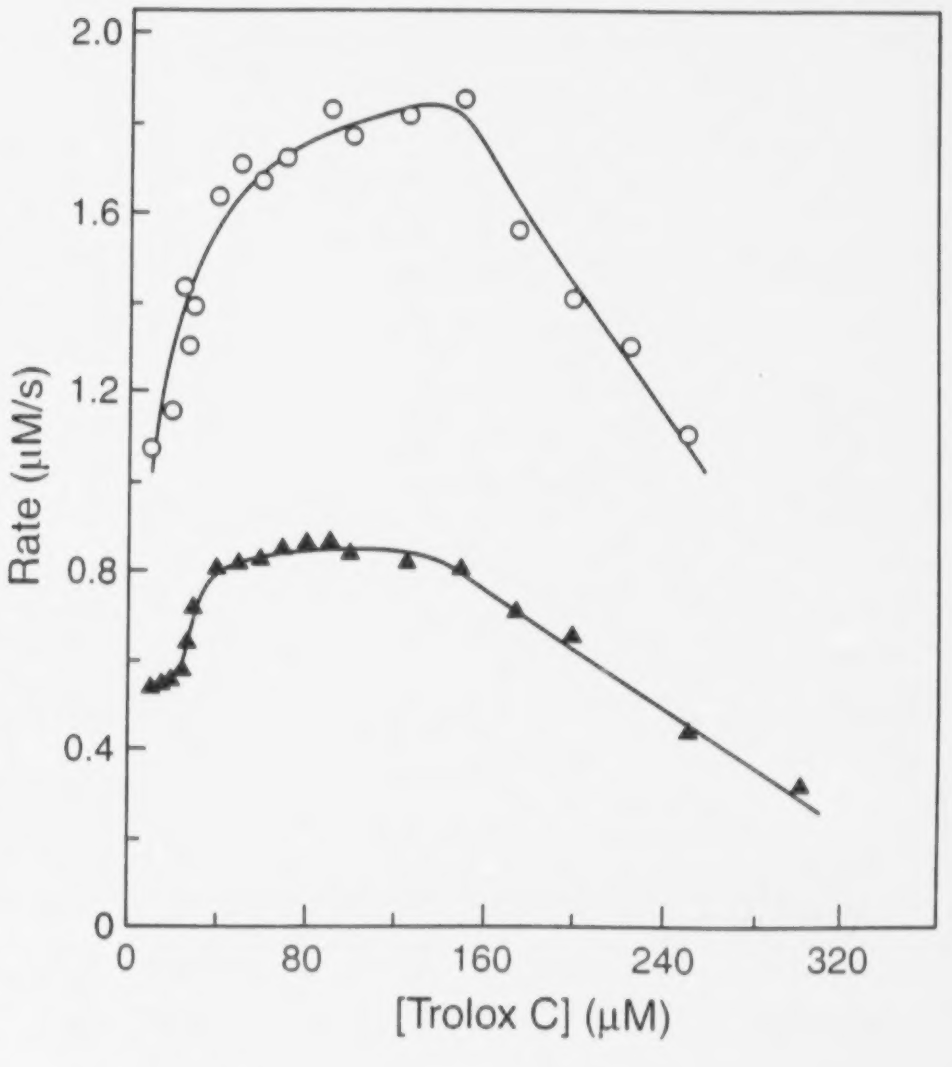


Figure 5. 8. Stimulatory and inhibitory effects of Trolox C on (▲) oxygen consumption and (O) conjugated diene formation during the reaction of 50 μM 20:2, 35 nM PGH synthase, 1 μM hematin and various amounts of Trolox C.

Table 5.2. Capacity of Trolox C and other electron donors for stimulating and inhibiting 20:2 oxygenation catalyzed by PGH synthase[a]

Electron	Oxygen consumption			Conjugated diene formation		
donor	Max Rate	A_{50}	I_{50}	Max Rate	A_{50}	I_{50}
	µM/s	µM	mM	µM/s	µM	mM
Trolox C	0.966	10.4	0.240	1.99	11.0	0.265
Phenol [b]	0.857	17.2	1.72			
DDC	1.12	2.21				
None [c]		K_m, µM			K_m, µM	
	1.17	69.7		1.98	34.5	

[a] Max rates, K_m, A_{50} and I_{50} values are obtained after fitting rectangular hyperbolae into the initial rate data. [b] Due to the spectral interferenes the rates of the conjugated diene formation were not measured in the presence of phenol and DDC; [c] Corresponding curves for the spontaneous reaction and the reaction in the presence of Trolox C are in Figures 5.5 and 5.8.

hematin. Under the same conditions phenol and DDC showed a similar increase in the maximum rates. The concentration of Trolox C required to produce one-half of the maximum stimulation in the rate of oxygen consumption and product formation, A_{50}, is 10.4-11.0 μM. The concentration of Trolox C necessary to reduce the rate of oxygen consumption and product formation to one-half of its maximum, I_{50}, is 0.240-0.265 mM. Trolox C appeared to be a better stimulator and inhibitor of oxygen consumption than phenol and poorer stimulator than DDC (Table 5.2).

Phenol and DDC spectra interfere with the conjugated diene spectra so that their effect on the conjugated diene formation could not measured. We obtained the initial rates for oxygen consumption and product formation by using the two different methods only for the Trolox C-affected oxygenase reaction, showing that A_{50} and I_{50} values are the same and independent on the method used (Table 5.2). This indicates that Trolox C identically affects oxygen consumption and conjugated diene product formation. In the absence of Trolox C, presumably in an oxygenase reaction alone, the kinetic parameters, maximum rates and K_m values, were approximately two times higher from the product formation measurements (stopped flow) than for the oxygen consumption (oxygen electrode) measurements (Table 5.2). These experiments are done by changing substrate 20:2 concentration, not at substrate fixed concentration as in the experiments with Trolox C.

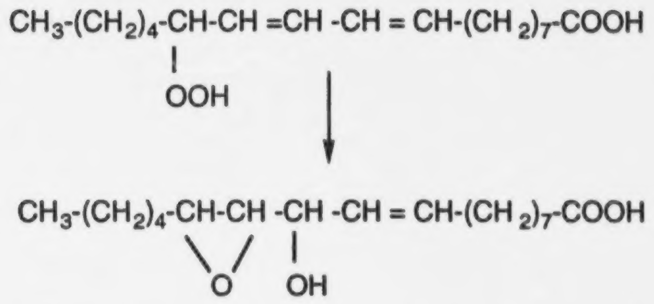
5.4. DISCUSSION

5.4.1. Initiation of Soybean Lipoxygenase and PGH Synthase Activities.

Two different mechanisms were proposed for the lipoxygenase reaction involving either (i) peroxyl radical formation (14, 15) or (ii) an intermediate (non radical) iron-alkyl complex formation (16). In any event, both of the lipoxygenase mechanisms require a peroxide for the initiation of enzymatic activity similarly to PGH synthase. For both enzymes the characteristics of the oxygenation reactions are (i) existence of the lag phase before the optimal oxygen consumption rate is reached, (ii) activation of the oxygen uptake by different peroxides, (iii) inhibition of the oxygen uptake by glutathione peroxidase, and (iv) loss of the enzyme activity in the presence of hydroperoxide alone (7, 17, 18).

The above evidence concerning the hydroperoxide involvement in the initiation of lipoxygenase and cyclooxygenase activity was obtained indirectly from polarographic measurements of the oxygen consumption rates. In this report we provide more direct evidence obtained by the stopped flow spectrophotometry for the role of contaminant hydroperoxide in the initiation of both lipoxygenase and PGH synthase reactions with fatty acids. It is readily apparent from Figures 5.2 and 5.3 that the initial (pre-steady state) drop in absorbance at 235 nm can only be attributed to a degradation of a conjugated diene hydroperoxide which had been spontaneously formed from 20:2. Higher enzyme or substrate concentrations diminished or eliminated the initial phase completely from the stopped flow kinetic traces. A question that needs to be answered is the following: why is there the initial drop in the absorbance if the contaminant hydroperoxide is reduced to the corresponding conjugated diene hydroxide? The absorbance at 235 nm specifically measures changes in the conjugation of the double bonds and the proposed change from hydroperoxide to hydroxide functional group should not make any significant difference in the absorbance (3).

A possible explanation can be found in the behaviour of lipoxygenase in its reaction with linoleic acid hydroperoxide. In the transition of the purple to yellow form of lipoxygenase (8) the linoleic acid hydroperoxide is transformed into an epoxy-hydroxy linoleic acid as follows:



$$\mathrm{CH_3\text{-}(CH_2)_4\text{-}\underset{OOH}{CH}\text{-}CH{=}CH\text{-}CH{=}CH\text{-}(CH_2)_7\text{-}COOH} \longrightarrow \mathrm{CH_3\text{-}(CH_2)_4\text{-}\underbrace{CH\text{-}CH}_{O}\text{-}\underset{OH}{CH}\text{-}CH{=}CH\text{-}(CH_2)_7\text{-}COOH}$$

This transition is accompanied by a loss of the conjugated system of double bonds. This suggests that a similar transformation of a conjugated diene hydroperoxide to an epoxyhydroxide could occur in the induction phase of the PGH synthase and soybean lipoxygenase catalyzed oxygenations of 20:2, which could explain the initial drops in the absorbance at 235.0 nm in the stopped flow kinetic traces in Figures 5.2 and 5.3.

5.4.2. Stoichiometry of the Soybean Lipoxygenase Reaction with 20:2.

The incorporation of one molecule of oxygen into the fatty acid in the lipoxygenase catalyzed oxygenation (8) explains the stoichiometry between oxygen consumption and product hydroperoxide formation of 1:1. Our experiments at both small (4.5 nM) and large (45.5 nM) concentrations of soybean lipoxygenase confirm this relationship. The measurements for the system soybean lipoxygenase/20:2 also represent a calibration test for our instruments. Since the correct stoichiometry for the lipoxygenase reaction was obtained, we conclude that there are no artifacts in the initial rates measurements with any of the three instruments used in this studies: a stopped flow spectrophotometer, a conventional spectrophotometer and an oxygen electrode.

5.4.3. Stoichiometry of the PGH Synthase Reaction with Eicosadienoic Acid

Based on previous studies we expected that the stoichiometric ratio between oxygen consumed and oxygenated product formed would not be the same in the presence and absence of peroxidase electron donors. Previous studies with radiolabelled 20:4 showed that phenol markedly affected the relative proportions of prostaglandin products (7). In the presence of phenol, the endoperoxide, PGG_2, was consumed rapidly and was subsequently converted to the hydroxide, PGH_2. In the absence of pretreatment with phenol or DDC the enzyme activity was low, but still some PGG_2 and PGH_2 were obtained, approximately in the same ratio (4, 5) or with PGG_2 as a predominant product (6, 7).

We chose to conduct the experiments with 20:2 because we could follow spectrophotometrically the initial rate of the oxygenation product formation, which is not possible with 20:4 as a substrate. We obtained quantitative conversion rates of 20:2 to the corresponding conjugated diene derivatives using stopped flow spectrophotometry. This method has advantages because it is fast, minimizes the effects of enzyme inactivation, and prevents the autooxidation which could explain the variations in the product ratios obtained by radioisotopic and chromatographic analyses.

The product spectra and shape of the kinetic traces are similar for both enzymes, but much to our surprise the stoichiometries are strikingly different. The ratio of the conjugated diene formed to molecular oxygen consumed is 1:1 for soybean lipoxygenase and 2:1 for PGH synthase. To our further surprise the 2:1 ratio for PGH synthase was not affected by the presence of Trolox C. If PGH synthase acted exclusively as an oxygenase (eq. 5.1) the expected conjugated diene product would be the hydroperoxide derivative and the ratio between the rates of oxygen consumed, $-d[O_2]/dt$ and hydroperoxide formed, $d[ROOH]/dt$ would be 1:1 as in the soybean lipoxygenase reaction. Furthermore, if the branched-chain tyrosyl radical mechanism for the PGH synthase-oxygenase reaction (19-21) were valid then the dominant product again is expected to be the hydroperoxide derivative and the stoichiometry between the hydroperoxide formed and the oxygen consumed would be 1:1. The 2:1 stoichiometry is not in agreement with formation of hydroperoxide only, with no alcohol formation, and consequently does not agree with the concept of an oxygenase reaction with no peroxidase reaction.

The fact that the rate of conjugated diene formation is double the rate of molecular oxygen consumption indicates a reaction mechanism more complex than a simple oxygenation reaction. Furthermore, hydroperoxide is known to be an excellent substrate for native PGH synthase, converting it into compound I with concomitant formation of the corresponding alcohol (22). We propose the following steady-state model which is in accord with all the known experimental facts.

5.4.4. Steady-State Model for the PGH Synthase Reaction with Eicosadienoic Acid

We propose a steady-state model for the oxygenation reaction of 20:2 catalyzed by PGH synthase which can account for (i) the requirement for hydroperoxide to initiate the reaction, (ii) hydroperoxide as a transient product, (iii) hydroxide as a main product, (iv) the stoichiometry between oxygen and oxygenation product, (v) a new role for the tyrosyl radical in the reaction mechanism, and (vi) the intimate relation between oxygenase and peroxidase functions of the enzyme.

The model includes features commonly attributed to cytochrome P-450 reaction: hydrogen atom abstraction from a hydrocarbon and oxygen atom insertion. The overall result is reaction of one molecule of oxygen and two molecules of 20:2 in order to form two molecules of hydroxy derivative of the acid:

$$\begin{array}{rcll}
\text{PGH synthase} + \text{ROOH} & \rightarrow & \text{ROH} + \text{Compound I} & [5.5] \\
\text{Compound I} + \text{RH} & \rightarrow & \text{R}\cdot + \text{Compound II} & [5.6] \\
\text{R}\cdot + \text{O}_2 & \rightarrow & \text{ROO}\cdot & [5.7] \\
\text{Compound II} + \text{ROO}\cdot & \rightarrow & \text{Compound I-Tyr}\cdot + \text{ROOH} & [5.8] \\
\text{Compound I-Tyr}\cdot + \text{RH} & \rightarrow & \text{PGH synthase} + \text{ROH} & [5.9] \\
\hline
2\ \text{RH} + \text{O}_2 & = & 2\ \text{ROH} & [5.10]
\end{array}$$

In the above model, hydroperoxide ROOH initiates the reaction by oxidizing native enzyme to Compound I in a two-electron transfer process and forming one molecule of hydroxide ROH (eq. 5.5). The fatty acid substrate RH can react with both Compound I and Compound I-Tyr· enzyme species. The reaction of fatty acid with Compound I goes first (eq. 5.6) and includes a one-electron and one-proton (hydrogen atom) transfer from fatty acid to Compound I. Peroxidase compound I is typically reduced by hydrogen atom donation (23) but not from a hydrocarbon. A fast addition of single molecule of oxygen to the fatty acid carbon radical, R· (formed in eq. 5.6), results in the formation of a hydroperoxyl radical ROO· (eq. 5.7). Compound II, formed in eq. 5.6 then donates one hydrogen atom to the hydroperoxyl radical forming Compound I-Tyr· and hydroperoxide ROOH (eq. 5.8). The reaction cycle is completed by the formation of a second molecule of hydroxide ROH (eq. 5.9) in the reaction of a second molecule of fatty acid with Compound I-Tyr· (eq. 5.9). This last step represents an oxygen insertion process similar to one proposed for the cytochrome P-450 reaction (24, 25).

The model does not require a participation of reducing substrate other than fatty acid in the reaction cycle and it does explain the existence of the spontaneous oxygenation reaction (Figure 5.1). It appears from our initial rate measurements that the reaction mechanisms for the stimulated and spontaneous oxygenation reactions catalyzed by PGH synthase are very much the same. In the previous studies (4-7) the hydroperoxide was isolated in the later phases of the reaction. We have shown that in the spontaneous reaction some additional oxidation of fatty acid occurs at longer times concomitant with an extensive bleaching of the enzyme (Figure 5.1). Thus the hydroperoxide production at longer time scales does not necessarily relate with the hydroperoxide production in the initial phases of the reaction when no significant enzyme bleaching occurs. There is no conflict between different investigations in the findings that hydroxide derivative is the only product obtained in the presence of electron donors in addition to fatty acid (5-7).

5.5.5. The Role of Trolox C in the PGH Synthase-Oxygenase Reaction

The stimulative role of the peroxidase electron donor Trolox C on the oxygenation of 20:2 may be to prevent the oxidative destruction of heme by the hydroperoxide (26, 27) which existed in the absence of Trolox C (inset to Figure 5.1). Trolox C can also facilitate the interconversion of some unproductive enzyme intermediates (20:2 is a poorer substrate for PGH synthase than 20:4) that can be formed during the cycle and thus promotes higher reaction rates without affecting the stoichiometry of the oxygenation of 20:2.

The role of the tyrosyl radical has been attributed not only to the catalysis but also to the auto-inactivation of the enzyme (26, 27). The tyrosyl radical formed in eq. 5.8 may be in an equilibrium with another protein radical, Compound I-X·, directly involved in a turnover-dependent oxidative inactivation (eq. 5.11):

$$\text{Compound I-Tyr}\cdot \rightleftarrows \text{Compound I-X}\cdot \qquad [5.11]$$

$$\text{Compound I-X}\cdot + \text{ROOH} \rightarrow \text{ROH} + H_2O + \text{Heme bleaching} \qquad [5.12]$$

A fraction of the enzyme will be inactivated (how much depends on the equilibrium constant for the step 5.11 and the rate constant for the step 5.12) and a portion of ROOH, formed in eq. 5.8, will be consumed during the process in eq. 5.12.

In the presence of peroxidase electron donor Trolox C both the enzyme inactivation and the hydroperoxide consumption are prevented (Eqs. 5.13 and 5.14):

$$\text{Compound I-X}\cdot + \text{Trolox C} \rightarrow \text{Compound II} + \text{Trolox C}\cdot \qquad [5.13]$$

$$\text{Compound II} + \text{Trolox C} \rightarrow \text{PGH Synthase} + \text{Trolox C}\cdot \qquad [5.14]$$

The unproductive protein radical is reduced to compound II and compound II to the native enzyme during the one-electron reductions with Trolox C. The enzyme activity which otherwise would be lost is restored and the amount of hydroperoxide formed (eq. 5.8) remains intact for the initiation of the next oxygenase cycle (eq. 5.5).

It is also possible that the reaction between the hydroperoxyl radical and compound II (eq. 5.8) is not highly specific; 20:2 is a poor substrate for PGH synthase, and a fraction of protein radical species different from the tyrosyl radical might be directly formed as in eq. 5.15:

$$\mathrm{ROO\cdot} + \text{Compound II} \rightarrow m\ \text{Compound I-Tyr}\cdot + n\ \text{Compound I-X}\cdot + \mathrm{ROOH} \qquad [5.15]$$

where $m + n = 1$. This reaction could be followed by the hydroperoxide-dependent oxidative inactivation in the absence of Trolox C (eq. 5.13), or the reductive stimulation in the presence of Trolox C (eqs. 5.13 and 5.14).

An unidentified protein radical different from the tyrosyl radical is involved in $\mathrm{Mn^{III}}$-PGH synthase cyclooxygenase and oxygenase reactions (28, 29). The findings that the tyrosyl radical can be involved in inactivation (27), and that an unknown protein radical can participate in PGH synthase catalysis (30) are consistent with our proposal for the existence of two protein radicals, one involved in catalysis and the other in inactivation. The two protein radicals may be in equilibrium (eq. 5.11), or are formed simultaneously (eq. 5.15). Their functions in the overall reaction of 20:2 can also be visualized in a way opposite from that represented in eqs. 5.8, 5.9, and 5.11-5.15, i.e., compound I-X· may participate in catalysis and compound I-Tyr· in the oxidative inactivation.

High concentrations of Trolox C inhibit the PGH synthase-oxygenase activity in a similar manner to high concentrations of other peroxidase electron donors (31), i.e. by competing with 20:2 for the fatty acid binding site and/or by reacting with enzyme species directly involved in the oxygenase reaction, compound I and compound I-Tyr·.

5.5.6. Conclusions

The combination of the polarographic and the stopped flow methods is a powerful tool for studying the stoichiometry and the overall reaction mechanism not only of 20:2 but also of structurally related compounds capable of forming a conjugated system of double bonds. The oxygenation of 20:2 by PGH synthase is initiated with a contaminant hydroperoxide as it is the oxygenation by soybean lipoxygenase but the stoichiometries and the overall catalytic mechanisms of the two reactions are qualitatively different.

The turnover-mediated oxidative damage of the heme prosthetic group of PGH synthase is prevented by Trolox C, a synthetic derivative of vitamin E. Vitamin E, as an integral component of cell membranes, could be a natural reducing substrate of PGH synthase, also a membrane component. Thus vitamin E may have a role similar to Trolox C i.e., to stimulate the fatty acid oxygenation by protecting the enzyme from the oxidative inactivation and preventing hydroperoxide consumption in destructive processes.

Further studies will be required to provide more insight into the catalytic mechanism of PGH synthase. The studies need to be extended to the pre-steady state and transient state regimes with the emphasis on the kinetics of the individual enzyme intermediates. In the following chapter we will show how a pre-steady state kinetic model for the cyclooxygenase and oxygenase reactions of PGH synthase is closely related to the steady-state model presented here. The previously suggested mechanism for the PGH synthase-cyclooxygenase reaction with 20:4, which includes the spontaneous formation of Compound I-tyrosyl radical from Compound I and a subsequent cyclooxygenase chain reaction (21, 30), does not explain the experimental results obtained neither here nor in the next chapter.

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CHAPTER SIX

PRE-STEADY STATE KINETICS AND MODELLING OF THE OXYGENASE AND CYCLOOXYGENASE REACTIONS OF PROSTAGLANDIN ENDOPEROXIDE SYNTHASE[*]

6.1. INTRODUCTION

The prostaglandin endoperoxide synthase (PGH synthase)[**] peroxidase reaction is a single elementary reaction in which an oxygen atom is transferred from the -OOH group of PGG_2 to native PGH synthase resulting in an iron(IV) porphyrin π-cation radical (compound I) and PGH_2 formation (1), a reaction typical of all heme containing peroxidases (2). The implication is that the so-called cyclooxygenase reaction occurs first (eq 6.1) and is followed by the peroxidase reaction (eq 6.2):

$$20{:}4 + 2\,O_2 \xrightarrow{\text{cyclooxygenase}} PGG_2 \qquad [6.1]$$

$$\text{PGH Synthase} + PGG_2 \xrightarrow{\text{peroxidase}} \text{Compound I} + PGH_2 \qquad [6.2]$$

On the other hand it is well known that for the initiation of oxygen consumption in the overall reaction, traces of a hydroperoxide are needed, and furthermore that classical peroxidase reducing substrates can cause dramatic changes in the rates of oxygen consumption, implying that the peroxidase function of the enzyme plays a key role in the cyclooxygenase reaction.

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[*] A version of this chapter has been submitted for publication in Biophysical Chemistry.

[**] Abbreviations: PGH synthase, prostaglandin endoperoxide synthase; PGG_2, prostaglandin G_2; PGH_2, prostaglandin H_2; 20:4, arachidonic acid; 20:4·, arachidonic acid carbon radical; PGG_2· , prostaglandin G_2 radical; 20:2, eicosadienoic acid; 22:6, docosahexaenoic acid; HOO-20:2, hydroperoxy eicosadienoic acid.

A tyrosyl-radical mechanism has been proposed for the cyclooxygenase reaction (3, 4). Compound I formed in eq 6.2 converts spontaneously by an intramolecular one-electron transfer to an iron(IV) tyrosyl radical (eq 6.3):

$$\text{Compound I} \longrightarrow \text{Compound I-Tyr}\cdot \qquad [6.3]$$

According to this hypothesis the tyrosyl radical then initiates the cyclooxygenase reaction as depicted in eqs 6.4-6.6:

$$\text{Compound I-Tyr}\cdot + 20{:}4 \longrightarrow \text{Compound II} + 20{:}4\cdot \qquad [6.4]$$

$$20{:}4\cdot + 2\,O_2 \longrightarrow PGG_2\cdot \qquad [6.5]$$

$$\text{Compound II} + PGG_2\cdot \longrightarrow \text{Compound I-Tyr}\cdot + PGG_2 \qquad [6.6]$$

Initially, the tyrosyl radical abstracts the hydrogen atom from 20:4 producing the carbon radical 20:4· and compound II (eq 6.4). The carbon radical is then trapped by two molecules of oxygen producing the $PGG_2\cdot$ radical (eq 6.5); and finally, PGG_2 is formed by hydrogen atom donation from a tyrosyl residue of compound II and the compound I-tyrosyl radical is regenerated (eq 6.6). The main implication of the tyrosyl radical mechanism is that once initiated, the branched-chain free radical reaction (eqs 6.4-6.6) can continue independently of the normal peroxidase cycle.

We showed in Chapter 4 that there is a fixed and constant (2:1) ratio between the rates of reaction of a conventional peroxidase reducing substrate ferulic acid (RH) and the cyclooxygenase substrate 20:4 that are incompatible with the branched-chain tyrosyl radical hypothesis. The corresponding mechanism is summarized in eqs. 6.2, 6.3a and 6.5-6.8:

$$\text{PGH Synthase} + \text{PGG}_2 \longrightarrow \text{Compound I} + \text{PGH}_2 \qquad [6.2]$$

$$\text{Compound I} + 20{:}4 \longrightarrow \text{Compound II} + 20{:}4\cdot \qquad [6.3a]$$

$$20{:}4\cdot + 2\text{O}_2 \longrightarrow \text{PGG}_2\cdot \qquad [6.5]$$

$$\text{Compound II} + \text{PGG}_2\cdot \longrightarrow \text{Compound I-Tyr}\cdot + \text{PGG}_2 \qquad [6.6]$$

$$\text{Compound I-Tyr}\cdot + \text{RH} \longrightarrow \text{Compound II} + \text{R}\cdot \qquad [6.7]$$

$$\text{Compound II} + \text{RH} \longrightarrow \text{PGH Synthase} + \text{R}\cdot \qquad [6.8]$$

Compound I is not converted spontaneously to the compound I-tyrosyl radical (eq 6.3). Compound I rather abstracts a hydrogen atom from 20:4 (eq. 6.3a) and forms compound II and the carbon radical 20:4•. The following two steps are the same as in the branched-chain tyrosyl radical mechanism (eqs. 6.5 and 6.6). The classical electron (hydrogen atom) donor RH (eqs. 6.7 and 6.8) reacts with both the compound I-tyrosyl radical and compound II and the native enzyme is regenerated.

The hypothetical role for the compound I-tyrosyl radical to initiate the oxygenation of fatty acids can be tested kinetically under pre-steady state conditions by following the rate of its formation at 426 nm, an isosbestic point between native enzyme and compound I, as a function of fatty acid concentration. The spontaneous formation of the compound I-tyrosyl radical from compound I (eq 6.3) is a unimolecular elementary reaction and the rate of its formation should be independent on the fatty acid concentration. Here we report that in the absence of classical peroxidase electron donors (the spontaneous cyclooxygenase reaction) the reaction in eq 6.3 does not occur. Rather, the disappearance of compound I is a function of fatty acid concentration as shown in eq 6.3a. The latter reaction was proposed previously for the combined cyclooxygenase-peroxidase reaction (Chapter 4; ref. 5). The compound I-tyrosyl radical (or electronically equivalent protein radical) is formed latter in a bimolecular reaction between the hydroperoxyl radical and compound II (eq. 6.6).

6.2. MATERIALS AND METHODS

6.2.1. Materials

All reagents used in this study were of the highest grade available. 20:4, 20:2, 22:6, indomethacin and hematin were purchased from Sigma; phenol from British Drug House; DE 53 ion-exchange chromatography gel from Whatman; Tween 20 from J. T. Baker. 20:4, 20:2 and 22:6 were dissolved in deaerated absolute ethanol and kept in small portions under nitrogen at -70°C. The aliquots of 20:2 stock solutions were dried with nitrogen and oxidized with oxygen for different periods of time (0-3 hours). After specific times, the excess of oxygen was removed with nitrogen and the oxidized samples of 20:2 were redissolved in deaerated absolute ethanol and kept at -70°C before use. The concentration of the conjugated diene hydroperoxide formed by the oxidation of 20:2 (HOO-20:2) was measured spectrophotometrically at 235 nm using an ε of 2.4×10^4 $M^{-1}cm^{-1}$ (6, 7).

PGH synthase was isolated from ram seminal vesicle microsomes using the published procedure (8, 9) described in Chapter 2. Measurements of protein content were conducted by the BioRad protein assay using bovine serum albumin as a standard. Enzyme concentrations were determined spectrophotometrically using an ε of 123 $mM^{-1}cm^{-1}$ (10).

6.2.2. Oxygen Electrode Measurements

Cyclooxygenase activity was determined by measuring oxygen consumption in the reaction of 20:4 with PGH synthase at 30°C in 0.1 M phosphate buffer, pH 8.0, using a Yellow Spring Instruments Model 53 oxygen monitor equipped with a Clark-type polarographic electrode. The oxygen concentration dissolved in the buffer was 230 mM at 30°C (11). The specific activity of the enzyme was calculated by assuming that 2 mol of oxygen were consumed per mole of 20:4. The enzyme preparations from two isolations were roughly a 30/70 mixture of holo- and apo-enzyme having a specific cyclooxygenase activity of 37 and 44 mM 20:4 mg protein^{-1}min^{-1} in the presence of 1 mM hematin and 1 mM phenol. The holo/apo enzyme preparations were reconstituted with hematin (2.5-fold more hematin was added than what was initially present in the holo/apo enzyme) immediately after isolation. The reconstituted enzyme was stored at -70°C without changing the heme content or enzyme activity during the experiments.

6.2.3. Rapid-Scan and Stopped-Flow Experiments

The experiments were performed on a Photal RA 601 Rapid Reaction Analyzer equipped with 1-cm cells thermostated at 30 ± 0.5 °C. The absorption spectra were measured by means of a multichannel photodiode array photometer, memorized in a computer system with the corresponding replica plotted on an Epson VP-550 recorder. Soret band absorption of the heme of PGH synthase was utilized for rapid scanning of the transformation of the enzyme species during the reactions.

Stopped-flow kinetic analysis was performed at a single wavelength from the Soret region, i.e., at 426 nm, an isosbestic point between the native enzyme and compound I (1, 4, 12, 13).

For the assays with 20:4, fixed amounts of PGH synthase (0.25 µM) and oxygen (230 µM) were reacted with different amounts of 20:4 (5.5-60 µM). The anaerobically stored stock solution of 20:4 contained trace amounts of hydroperoxide capable of activation of the cyclooxygenase reaction. Any change in [20:4] in the assay mixture automatically caused changes in the hydroperoxide concentration.

To overcome the difficulty of not being able to control hydroperoxide concentration, we performed a different type of experiment with 20:2. A series of stock solutions containing various amounts of 20:2 and hydroperoxide HOO-20:2 were prepared by oxidizing 20:2 samples with oxygen. Thus, it was possible to keep the [HOO-20:2] constant while the [20:2] was varied. Single assays under pseudo-first order conditions were carried out at constant concentration of enzyme (0.25 μM), oxygen (230 μM) and hydroperoxide (2.5 μM), and varied concentrations of 20:2 (5.3-99 μM).

The rest of the procedure was identical for both 20:4 and 20:2. One cell of the Rapid Reaction Analyzer contained PGH synthase dissolved in 0.1 M phosphate buffer, pH 8.0. The enzyme solution was incubated 3 min at 30°C before reaction. The other cell contained the fatty acid/hydroperoxide mixture dissolved in the same buffer. The buffer solution was also preincubated for 3 min at 30°C and fatty acid/hydroperoxide were added to the cell immediately before measurements. Mixing of solutions to cause the reaction was complete in 2 ms. Duplicate or triplicate sets of experiments were performed for identical reaction mixtures and usualy 5-8 reproducible kinetic traces were collected for each set. The relative standard deviation was in the range of 7-10%.

6.2.4. Data analysis

The observed pseudo-first order rate constants, k_{obs}, obtained from the exponential curve fits to the kinetic traces at 426 nm were plotted as a function of [fatty acid]. Different mechanistic models were simulated on a Macintosh Plus 40SC computer. A system of first-order differential equations was defined and numerically integrated by the 4th order Runge-Kutta method using the GRAF

PLOT general plotting program kindly provided by Dr. R. B. Jordan, University of Alberta. Numerical integration using the GRAF PLOT program was conducted by an iterative process, varying one rate constant at a time. The ranges of the rate constants were assigned based on the experimental values obtained in this study and on those already published (3, 4 , 12). The k_{obs} values were determined from the computed kinetic parameters and compared with those obtained experimentally.

6.2.5. Experiments with Indomethacin and 22:6

The effect of the cyclooxygenase inhibitors indomethacin and 22:6 on the values of the observed pseudo-first order rate constant, k_{obs}, was monitored at constant concentrations of enzyme (0.25 µM), oxygen (230 µM) and 20:4 (50 µM), and various concentrations of indomethacin or 22:6. In one cell of the stopped flow apparatus the enzyme and inhibitor were incubated 2 min in 0.1 M phosphate buffer, pH 8.0, at 30°. In the other, 20:4 was added immediately before measurements. Reproducible kinetic traces were obtained within the next 2 min. If the times for the measurements were prolonged and/or not controlled the traces were not very reproducible because of time-dependent inhibitory processes, an irreversible inhibition with indomethacin and possibly the autooxidation of 22:6 (14-17).

6.3. RESULTS

6.3.1. Spectral Changes during the Cyclooxygenase and Oxygenase Reactions

In preliminary experiments with 20:4 as a substrate (the cyclooxygenase reaction) we obtained identical changes in the enzyme Soret spectra as were obtained previously (1, 4, 12, 13). Typically, intermediate II (compound II or compound I-Tyr·) was formed in a mixture with native enzyme. The native enzyme has an absorption maximum at 410 nm. Initially there is an absorbance decrease and an isosbestic point at 420 nm appears indicating the presence of a steady-state mixture of intermediate II and native enzyme. The spectrum usually becomes complicated with heme bleaching at higher fatty acid concentrations and/or at longer times. Figure 6.1 shows, for the first time, changes in the PGH synthase Soret spectra obtained for the oxygenase reaction with 20:2. The spectral changes are qualitatively the same as in the cyclooxygenase reaction with 20:4. As the existence of the isosbestic point at 420 nm indicates, a mixture of native enzyme and intermediate II was present. This reaction was conducted semi-anaerobically with oxygen partially removed from the buffer before a 1:1 ratio of the enzyme and HOO-20:2 (0.25μM/0.25μM) and an excess of 20:2 (37 μM) were introduced to initiate the reaction. Under these conditions the oxygen is a limiting reagent and the reaction was slowed down to the level at which it was possible to apply the conventional spectroscopy to obtain the changes in the enzyme spectra; no bleaching of the heme occured during 2 min. This experiment shows that the PGH synthase spectral intermediates can be obtained not only at low temperatures with fast techniques (1, 4, 12, 13) but also at room temperature with conventional methods if lower amounts of oxygen and hydroperoxide are used.

The rapid-scan method was used in a routine control of the experimental conditions before the stopped-flow kinetic measurements were performed. In these experiments the oxygen concentration (230 μM dissolved in the buffer at 30°C) was not changed and the hydroperoxide was in excess of the enzyme. Except that the reactions were faster, they resulted in no principal difference from the spectra shown in Figure 6.1.

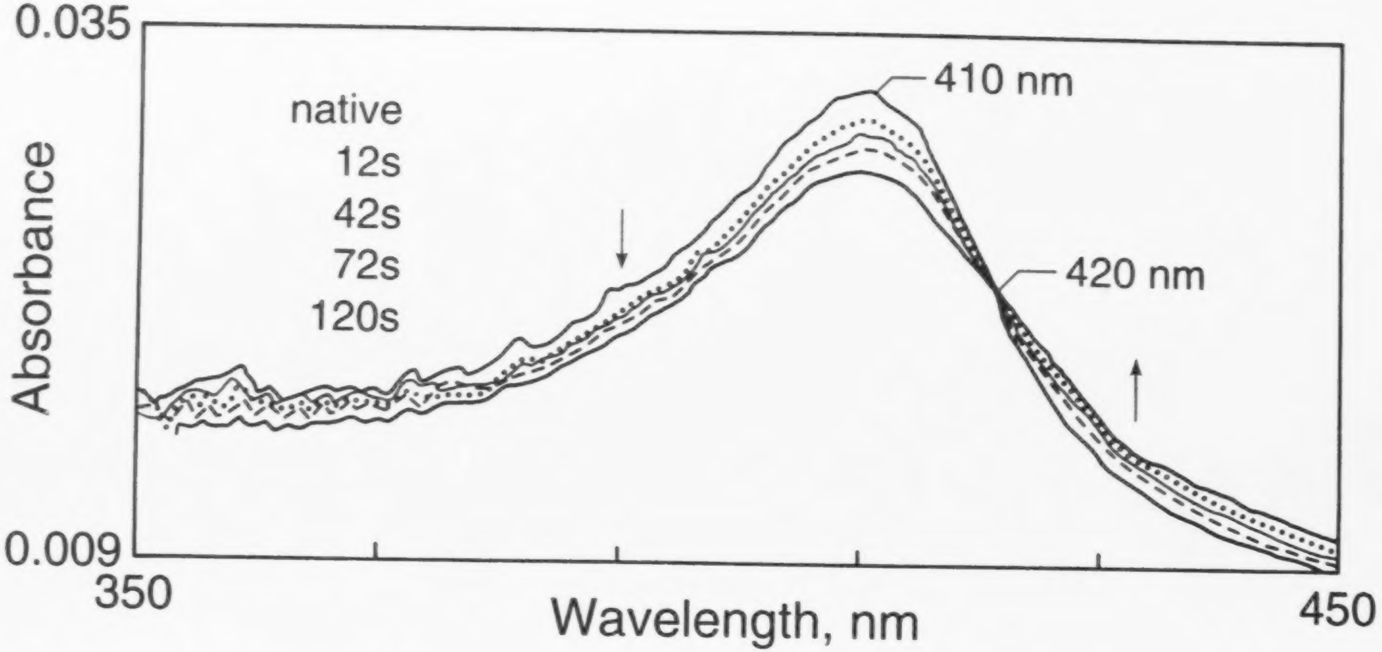

Figure 6.1. PGH synthase optical spectra recorded during the oxygenase reaction. The reaction was carried out in 0.1 M phosphate buffer, pH 8.0, at 22ºC. Before the enzyme (0.25 µM), 20:2 (37 µM) and HOO-20:2 (0.25 µM) were mixed O_2 was partially removed from the buffer by N_2.

6.3.2. Effects of 20:4 and 20:2 on the Intermediate II Formation

The isosbestic point between the native enzyme and compound I at 426 nm has been clearly identified under transient-state conditions for the peroxidase reactions of native enzyme with hydroperoxides (1, 4). The 426 nm wavelength has been used for monitoring the conversion of compound I to compound I-Tyr· (4) in a reaction initiated by PGG_2 and with no fatty acid present. We followed the same reaction as a function of [fatty acid]. A dependence of the rate of the formation of intermediate II from [fatty acid] was obtained with both 20:4 and 20:2 as substrates. Figure 6.2 shows kinetic traces obtained with 20:4. At the lowest [20:4] the kinetic trace shows a pre-steady state phase in which there is an induction period (lag phase) before the intermediate II started to form (Figure 6.2A). At longer times the steady-state is established. The presence of the lag phase indicates that yet another intermediate (compound I) was formed before the intermediate II. If the [fatty acid] is higher the lag phase is diminished but does not disappear from the kinetic traces (Figure 6.2B and C). The dependence of the rate of formation of intermediate II on [fatty acid] would not be expected for the unimolecular formation of compound I-Tyr·, which would have a spectrum identical to that of compound II (eq. 6.3) (4). Both the induction phase and the pre-steady state phase were faster for 30 and 60 μM 20:4 than at 5.5 μM 20:4, indicating that intermediate II could be a conventional compound II but not compound I-Tyr·.

The stopped-flow and rapid-scan studies of the reaction of PGH synthase with different [20:4] were extended to longer times (50 s) after the steady-state conditions had been established (data not shown). These experiments show that the return of intermediate II to the native state (partly also shown in Figure 6.2C as a drop in absorbance after the steady state has been reached) is complicated by enzyme bleaching. Thus, the inactivation events occur after, not before,

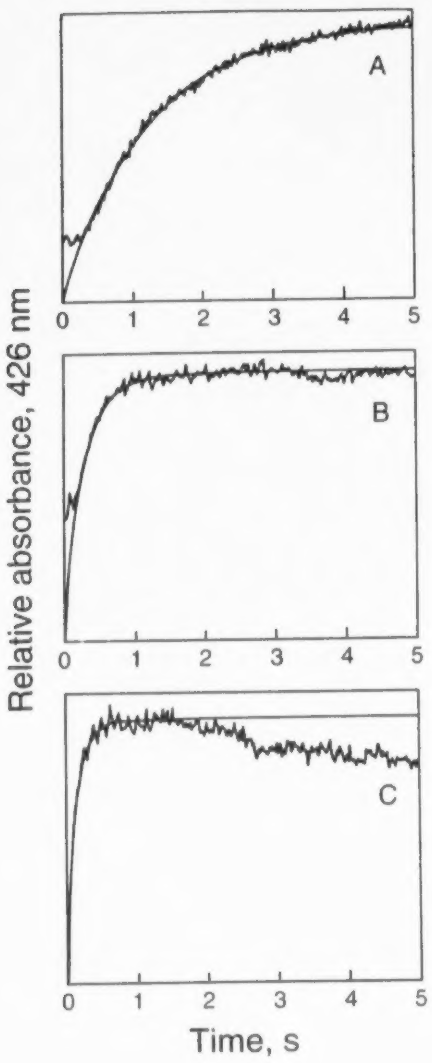

Figure 6.2. Effect of [20:4] on the rates of the intermediate II formation. Absorbance changes were followed at 426 nm, an isosbestic point between native enzyme and compound I. **A.** 5.5, **B.** 30, **C.** 60 μM of 20:4 reacted with 0.25 μM PGH synthase in 0.1 M phosphate buffer, pH 8.0, at 30°C. The k_{obs} is: **A.** 0.813, **B.** 3.50, and **C.** 7.36 s^{-1}.

intermediate II is formed. We were not interested in the inactivation process; we followed the events before the steady-state conditions were established.

6.3.3. Inhibition of the Intermediate II Formation

The cyclooxygenase inhibitors indomethacin and 22:6 inhibited the rate of the intermediate II formation as indicated in Figure 6.3. The concentration of indomethacin necessary to cause a 50% decrease of the observed pseudo-first order rate constant, k_{obs}, is 2.3 µM when the reaction was performed with 50 µM 20:4 and 0.25 µM enzyme. The fatty acid 22:6 is a significantly weaker inhibitor of the intermediate II formation than indomethacin. No linear decrease in k_{obs} as a function of [22:6] was obtained; k_{obs} leveled off at 20-60 µM of [22:6] at a value approximately 44% lower than in the absence of 22:6.

6.3.4. Kinetic Model for the Oxygenase Reaction

The observed pseudo-first order rate constant, k_{obs}, obtained from the pre-steady state kinetic measurements was a linear function of [20:2], a characteristic typical of bimolecular reactions (Figure 6.4). The experiments were performed at constant [PGH synthase] and 10-fold higher [HOO-20:2]. Only [20:2] was varied in the reaction mixtures. The experimental conditions were essentially pseudo-first order and single exponential curves were obtained at 426 nm. Analysis of the [20:2] dependence of the pre-steady state kinetics led to the following proposal of a mechanism of the oxygenase pathway of PGH synthase.

Scheme 6.1:

$$\text{E} + \text{AOOH} \xrightarrow{k_1} \text{E-I} + \text{AOH} \qquad [6.9]$$

$$\text{E-I} + \text{AH} \xrightarrow{k_2} \text{E-II} + \text{A}\cdot \qquad [6.10]$$

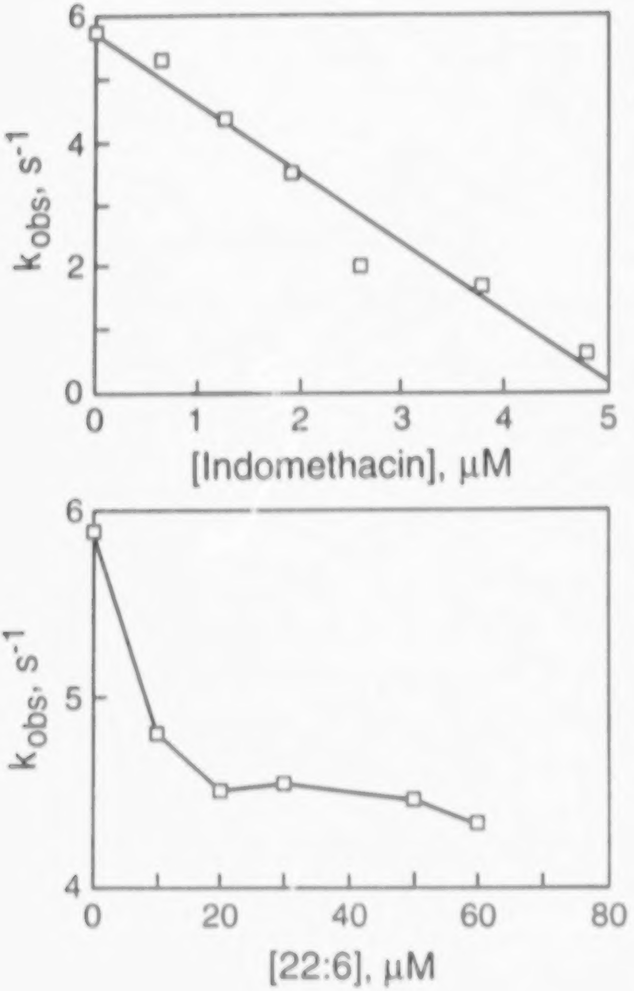

Figure 6. 3. Effects of cyclooxygenase inhibitors on the observed pseudo-first order rate constant, k_{obs}, for the reaction of 50 μM 20:4 and 0.25 μM enzyme. **A.** 0-4.8 μM indomethacin and **B.** 0-60 μM 22:6.

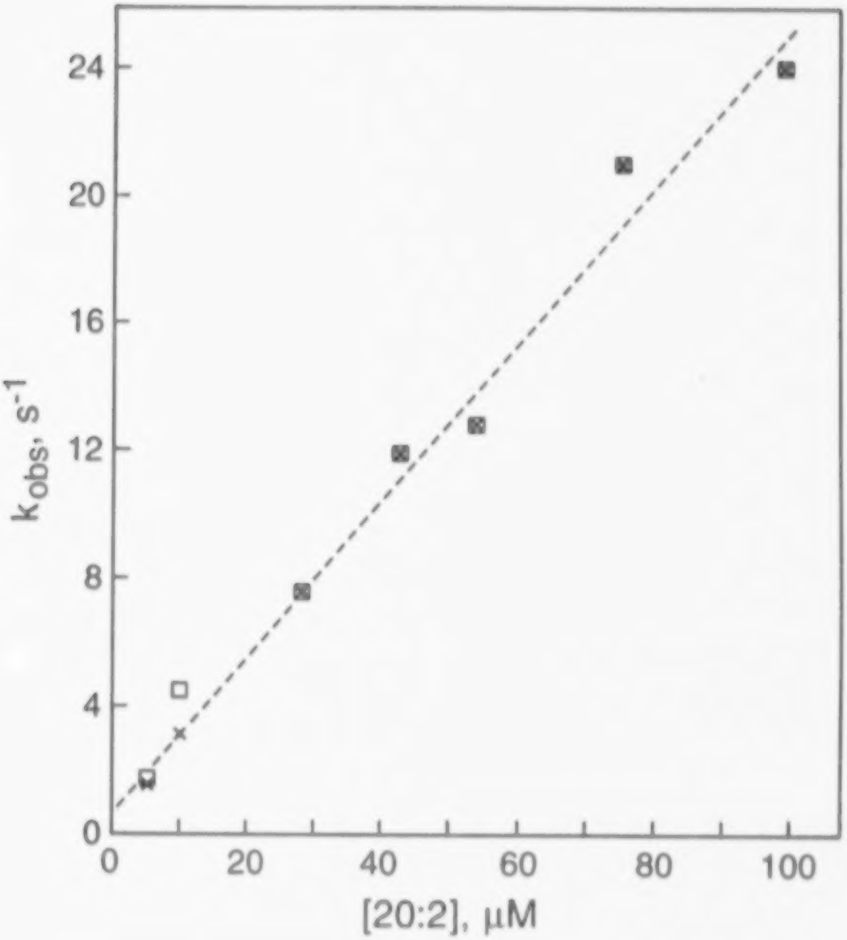

Figure 6. 4. Comparison of the experimental (□) and simulated (x) k_{obs} versus [20:2]. Theoretical k_{obs} were calculated according to the model in Scheme 6.1 by using the following kinetic parameters: $k_1 = 4 \times 10^7$ M^{-1} s^{-1}; $k_2 = 3 \times 10^5$ M^{-1} s^{-1} ; and $k_3 = 3 \times 10^{-3}$ s^{-1}. Initial conditions: $[E]_o = 0.25$ μM, $[AH]_o = 5.3$-90 μM, $[AOOH]_o = 2.5$ μM.

$$\mathrm{A\cdot + O_2 \xrightarrow{k_{O_2}} AOO\cdot} \qquad [6.11]$$

$$\mathrm{E\text{-}II + AOO\cdot \xrightarrow{k_{AOO}} E\text{-}I\text{-}X\cdot + AOOH} \qquad [6.12]$$

$$\mathrm{E\text{-}I\text{-}X\cdot \xrightarrow{k_3} E} \qquad [6.13]$$

The above model predicts the existence of three enzyme intermediates during the oxygenase reaction, compound I (E-I), compound II (E-II) and compound I-protein radical (E-I-X·). All steps are bimolecular except the last one: native enzyme (E) reacts with fatty acid hydroperoxide AOOH, compound I (E-I) with fatty acid AH, and compound II (E-II) with fatty acid hydroperoxyl radical AOO·. A unimolecular last step was assumed for the spontaneous decay of the protein radical E-I-X· to the native enzyme. This step is a secondary reaction which occurs at much lower rates then the formation of the intermediates.

A set of seven differential equations corresponding to the mechanism in Scheme 6.1. is defined as follows:

$$d[\mathrm{E}]/dt = k_3\,[\mathrm{E\text{-}I\text{-}X\cdot}] - k_1\,[\mathrm{E}]\,[\mathrm{AOOH}] \qquad [6.14]$$

$$d[\mathrm{E\text{-}I}]/dt = k_1\,[\mathrm{E}]\,[\mathrm{AOOH}] - k_2\,[\mathrm{E\text{-}I}]\,[\mathrm{AH}] \qquad [6.15]$$

$$d[\mathrm{E\text{-}II}]/dt = k_2\,[\mathrm{E\text{-}I}]\,[\mathrm{AH}] - k_{AOO\cdot}\,[\mathrm{E\text{-}II}]\,[\mathrm{AOO\cdot}] \qquad [6.16]$$

$$d[\mathrm{E\text{-}I\text{-}X\cdot}]/dt = k_{AOO\cdot}\,[\mathrm{E\text{-}II}]\,[\mathrm{AOO\cdot}] - k_3\,[\mathrm{E\text{-}I\text{-}X\cdot}] \qquad [6.17]$$

$$d[\mathrm{A\cdot}]/dt = k_2\,[\mathrm{E\text{-}I}]\,[\mathrm{AH}] - k_{O_2}\,[\mathrm{A\cdot}]\,[\mathrm{O_2}] \qquad [6.18]$$

$$d[\mathrm{AOO\cdot}]/dt = k_{O_2}\,[\mathrm{A\cdot}]\,[\mathrm{O_2}] - k_{AOO\cdot}\,[\mathrm{E\text{-}II}]\,[\mathrm{AOO\cdot}] \qquad [6.19]$$

$$d[\mathrm{AOOH}]/dt = k_{AOO\cdot}\,[\mathrm{E\text{-}II}]\,[\mathrm{AOO\cdot}] - k_1\,[\mathrm{E}]\,[\mathrm{AOOH}] \qquad [6.20]$$

By setting differential eqs. 18 and 19 to zero, one can find the values of the steady-state concentrations of the fatty acid carbon radical A· and the fatty acid hydroperoxyl radical AOO· :

$$[A\cdot] = \frac{k_2[E\text{-}I][AH]}{k_{O2}[O_2]^2} \tag*{[6.21]}$$

$$[AOO\cdot] = \frac{k_2[E\text{-}I][AH]}{k_{AOO\cdot}[E\text{-}II]} \tag*{[6.22]}$$

Substituting [A·] and [AOO·] in eqs. 6.16, 6.17 and 6.20 the model is reduced to four differential equations as follows:

$$d[E]/dt = k_3[E\text{-}I\text{-}X\cdot] - k_1[E][AOOH] \tag*{[6.23]}$$

$$d[E\text{-}I]/dt = k_1[E][AOOH] - k_2[E\text{-}I][AH] \tag*{[6.24]}$$

$$d[E\text{-}I\text{-}X\cdot]/dt = k_2[E\text{-}I][AH] - k_3[E\text{-}I\text{-}X\cdot] \tag*{[6.25]}$$

$$d[AOOH]/dt = k_2[E\text{-}I][AH] - k_1[E][AOOH] \tag*{[6.26]}$$

Assuming that A· and AOO· are under steady state conditions the d[E-II]/dt (eq 6.16) becomes zero automatically.

Figure 6.5 shows some possible solutions for the oxygenase reaction calculated by the model in Scheme 6.1 by using different combinations of the rate constants k_1, k_2 and k_3. The rate constants were changed one at a time. The calculated kinetic trace is most sensitive to the value of k_2 (Figure 6.5, c and d), moderately sensitive to k_1 (Figure 6.5, e) and least sensitive to k_3 (Figure 6.5, a and b). The rate constant k_2 seriously affects the shape of the whole trace; k_1 mostly affects the exponential part of the trace (the pre-steady state phase) while k_3 predominantly influences the amplitude of the trace (the steady-state phase).

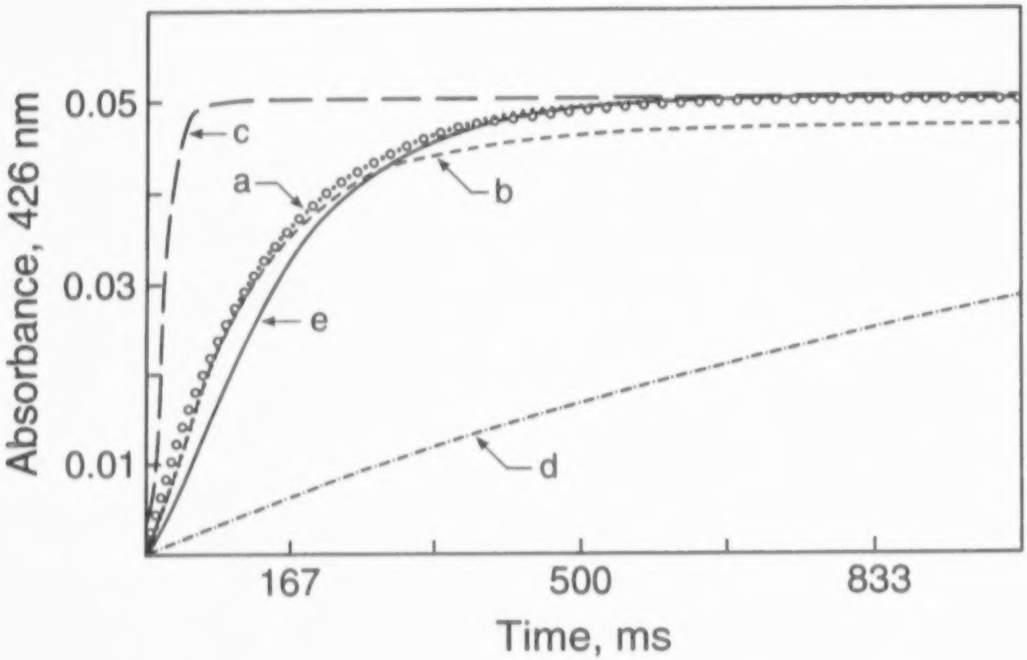

Figure 6.5. Sensitivity of the kinetic model proposed for the oxygenase reaction on the kinetic rate constants k_1, k_2 and k_3, according to the pathway in Scheme 6.1. (o) the experimentally obtained exponential curve with $k_{obs} = 7.62\ s^{-1}$ **(a)** $k_1 = 4 \times 10^7\ M^{-1}s^{-1}$, $k_2 = 3 \times 10^5\ M^{-1}s^{-1}$, $k_3 = 5 \times 10^{-3}\ s^{-1}$; **(b)** $k_1 = 4 \times 10^7\ M^{-1}s^{-1}$, $k_2 = 3 \times 10^5\ M^{-1}s^{-1}$, $k_3 = 0.5\ s^{-1}$; **(c)** $k_1 = 4 \times 10^7\ M^{-1}s^{-1}$, $k_2 = 3 \times 10^6\ M^{-1}s^{-1}$, $k_3 = 5 \times 10^{-3}\ s^{-1}$; **(d)** $k_1 = 4 \times 10^7\ M^{-1}s^{-1}$, $k_2 = 3 \times 10^4\ M^{-1}s^{-1}$, $k_3 = 5 \times 10^{-3}\ s^{-1}$; **(e)** $k_1 = 1 \times 10^6\ M^{-1}s^{-1}$, $k_2 = 3 \times 10^5\ M^{-1}s^{-1}$, $k_3 = 5 \times 10^{-3}\ s^{-1}$; $[E]_o = 0.25\ \mu M$, $[AH]_o = 28.3\ \mu M$, and $[AOOH]_o = 2.5\ \mu M$

The observed exponential increase in absorbance at 426 nm (Figure 6.6A) was fully reproduced by the model as the increase in the concentration of E-I-X· species (Figure 6.6B). Changes in concentrations against time for the other two main species E, E-I are also shown in Figure 6.5B. The best fit for this and other experiments with 20:2 were obtained with $k_1 = 4 \times 10^7\ M^{-1}s^{-1}$, $k_2 = 3 \times 10^5\ M^{-1}s^{-1}$ and $k_3 = 3 \times 10^{-3}\ s^{-1}$. The computer simulated and experimentally obtained k_{obs} are plotted as a function of [20:2] in Figure 6.4.

6.3.5. Kinetic Models for the Cyclooxygenase Reaction

The experimental plot shown in Figure 6.7 demonstrates that there is a similar linear relationship between k_{obs} and [20:4] for the cyclooxygenase reaction as for the oxygenase reaction (Figure 6.4) . However, the principal difference between the two studies was that in the cyclooxygenase reaction the initial concentration of hydroperoxide was not constant. Due to the presence of an unknown amount of a contaminant hydroperoxide in stock solutions of 20:4, whenever the initial [20:4] was changed the initial [hydroperoxide] also changed.

An experimentally obtained kinetic trace for the cyclooxygenase reaction and the best simulated trace for the same reaction are shown in Figure 6.8. The simulated trace was obtained according to the mechanism which predicts that compound I reacts with 20:4 and thus initiates the cyclooxygenase reaction as follows:

Scheme 6.2:

$$\text{E} + \text{A(O}_2\text{)OOH} \xrightarrow{k_1} \text{E-I} + \text{A(O}_2\text{)OH} \qquad [6.27]$$

$$\text{E-I} + \text{AH} \xrightarrow{k_2} \text{E-II} + \text{A}\cdot \qquad [6.28]$$

$$\text{A}\cdot + 2\,\text{O}_2 \xrightarrow{k_{O_2}} \text{A(O}_2\text{)OO}\cdot \qquad [6.29]$$

$$\text{E-II} + \text{A(O}_2\text{)OO}\cdot \xrightarrow{k_{AOO\cdot}} \text{E-I-X}\cdot + \text{A(O}_2\text{)OOH} \qquad [6.30]$$

$$\text{E-I-X}\cdot \xrightarrow{k_3} \text{E} \qquad [6.31]$$

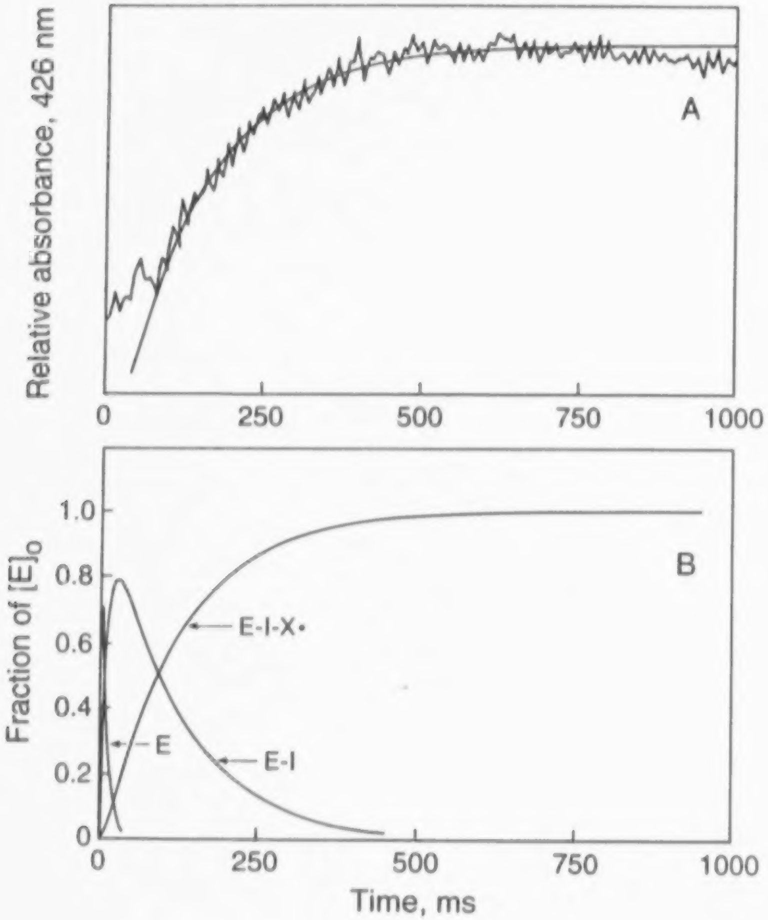

Figure 6.6. Absorbance changes and curve fit of the kinetic trace at 426 nm **(A)** and simulated time courses **(B)** of the enzyme species during the reaction of PGH synthase and 20:2. **(A)** Conditions: 28.3 μM 20:2, 2.5 μM HOO-20:2 and 0.25 μM enzyme ($[E]_o$). **(B)** The time profiles for native enzyme (E), compound I (E-I) and compound I-protein radical (E-I-X·) calculated by numerical integration according to the model shown in Scheme 6.2 with the following rate constants $k_1 = 4 \times 10^7$ and $k_2 = 3 \times 10^5\ M^{-1}\ s^{-1}$; $k_3 = 3 \times 10^{-3}\ s^{-1}$ and $k_{obs} = 7.62\ s^{-1}$

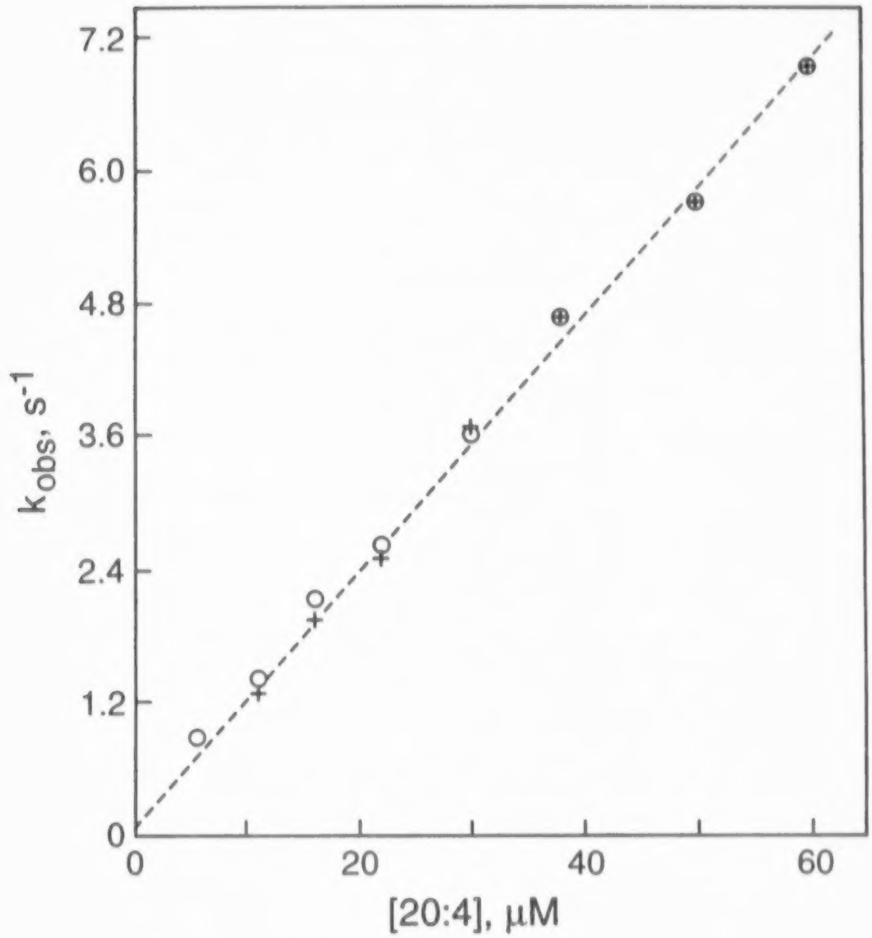

Figure 6.7. Experimental (o) and simulated (+) data showing the linear dependence of k_{obs} from [20:4]. Theoretical k_{obs} were calculated according to the kinetic model in Scheme 6.2 by using the following rate constants $k_1 = 8 \times 10^7\ M^{-1}s^{-1}$, $k_2 = 1.2 \times 10^5\ M^{-1}s^{-1}$, and $k_3 = 3 \times 10^{-3}\ s^{-1}$ and initial conditions: $[E]_o = 0.25\ \mu M$, $[AH]_o = 5.5\text{-}60\ \mu M$.

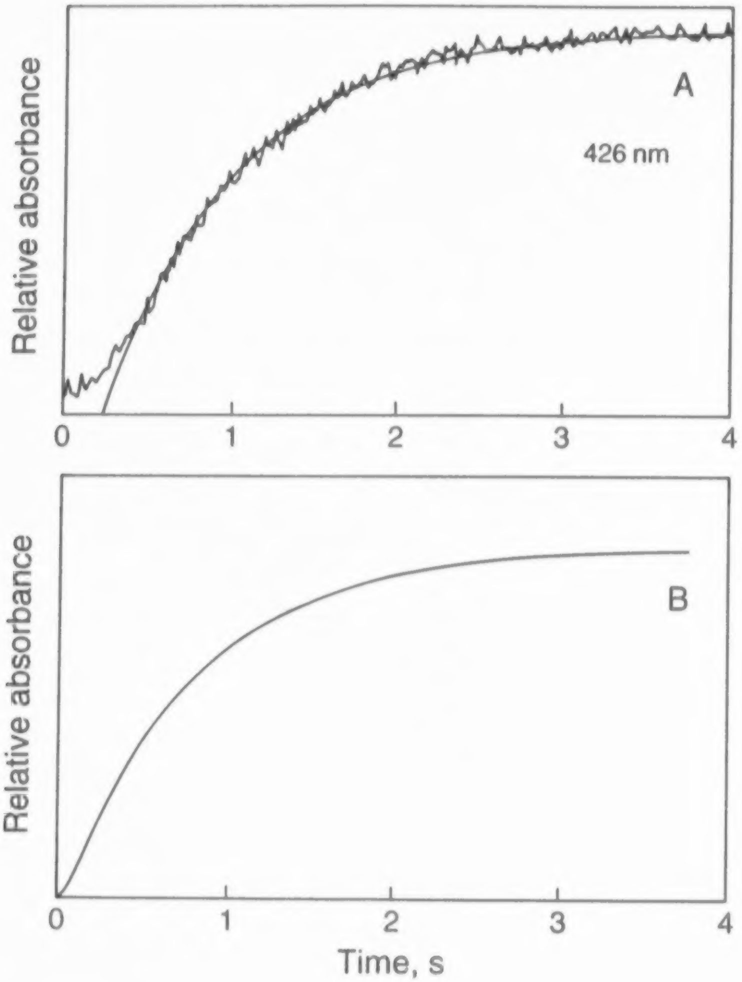

Figure 6.8. Absorbance change and curve fit of the kinetic trace at 426 nm **(A)** and simulated time course **(B)** during the reaction of PGH synthase and 20:4. **A**. The reaction mixture contained 11 μM 20:4 and 0.25 μM enzyme. **B.** The time profile for compound I-protein radical (E-I-X·) calculated by numerical integration according to the model in Scheme 6.2 with the following rate constants $k_1 = 8 \times 10^7$ and $k_2 = 1.2 \times 10^5\ M^1 s^{-1}$; $k_3 = 3 \times 10^{-3}\ s^{-1}$ and $k_{obs} = 1.27\ s^{-1}$ and initial conditions: $[AH]_o = 11$ μM , $[A(O_2)OOH]_o = 0.39$ μM, and $[E]_o = 0.25$ μM.

In Scheme 6.2, $A(O_2)OOH$ and $A(O_2)OH$ are PGG_2 (hydroperoxide-endoperoxide) and PGH_2 (hydroxy-endoperoxide), and AH is 20:4. The mechanism for the cyclooxygenase reaction is identical to that for the oxygenase reaction (Scheme 6.1) except that two molecules of oxygen are necessary to produce the hydroperoxide endoperoxide radical $A(O_2)OO\cdot$. The mechanism in Scheme 6.2 is defined by a similar set of differential equations as in Scheme 6.1. After applying the steady-state approximation for $[A\cdot]$, $[A(O_2)OO\cdot]$ and [E-II] the cyclooxygenase system is governed by an identical set of working equations (eqs. 6.23-6.26) as the oxygenase system.

For the best simulation shown in Figure 6.8B we assigned the values for the rate constant k_1 of $8 \times 10^7\ M^{-1}\ s^{-1}$, based on the reported value for a similar 20:4-hydroperoxide, 15-hydroperoxy eicosatetraenoic acid (15-HPETE) of $7.1 \times 10^7\ M^{-1}\ s^{-1}$ at 25 °C (14), for k_2 $1.2 \times 10^5\ M^{-1}s^{-1}$ and $k_3 = 3 \times 10^{-3}\ s^{-1}$. We estimated the initial hydroperoxide concentration while keeping other parameters constant. It appeared that the best estimate for the hydroperoxide concentration was 3.4-3.5% of the initial [20:4]. This approximates the amount of hydroperoxide initially present in the 20:4 stock solutions. According to the manufacturer the purity of 20:4 was 99% but we knew from the spectroscopic measurements with similarly sensitive 20:2 (99% purity) that some autooxidation occurs during the storage and the preparation of the stock solutions leading to the initial presence of 2-3% of HOO-20:2 in 20:2 stock solutions. The theoretical and experimental plots of k_{obs} against [20:4] obtained for a series of [20:4] were identical when the above conditions were applied (Figure 6.7).

The second mechanism tested, shown below in Scheme 6.3, is based on the tyrosyl-radical hypothesis in which compound I-tyrosyl radical reacts with 20:4 and thus initiates the cyclooxygenase cycle. Instead of specifying the nature of the intermediate as the compound I-tyrosyl radical we would rather call it the compound I-protein radical (E-I-X·); no electronic nor spectroscopic differences in the heme part of the two possible intermediates exists and it does not make any principal change in the mechanism.

Scheme 6.3:

$$\mathrm{E} + \mathrm{A(O_2)OOH} \xrightarrow{k_1} \mathrm{E\text{-}I} + \mathrm{A(O_2)OH} \qquad [6.32]$$

$$\mathrm{E\text{-}I} \xrightarrow{k_{2a}} \mathrm{E\text{-}I\text{-}X}\cdot \qquad [6.33]$$

$$\mathrm{E\text{-}I\text{-}X}\cdot + \mathrm{AH} \xrightarrow{k_2} \mathrm{E\text{-}II} + \mathrm{A}\cdot \qquad [6.34]$$

$$\mathrm{A}\cdot + 2\,\mathrm{O_2} \xrightarrow{k_{O_2}} \mathrm{A(O_2)OO}\cdot \qquad [6.35]$$

$$\mathrm{E\text{-}II} + \mathrm{A(O_2)OO}\cdot \xrightarrow{k_{AOO\cdot}} \mathrm{E\text{-}I\text{-}X}\cdot + \mathrm{A(O_2)OOH} \qquad [6.36]$$

$$\mathrm{E\text{-}I\text{-}X}\cdot \xrightarrow{k_3} \mathrm{E} \qquad [6.37]$$

As in the first model the initial set of equations corresponding to the mechanism in Scheme 6.3 has been reduced to four equations by applying the steady-state approximation for A·, A(O_2)OO· and E-II:

$$d[\mathrm{E}]/dt = k_3\,[\mathrm{E\text{-}I\text{-}X}\cdot] - k_1\,[\mathrm{E}]\,[\mathrm{AOOH}] \qquad [6.38]$$

$$d[\mathrm{E\text{-}I}]/dt = k_1\,[\mathrm{E}]\,[\mathrm{AOOH}] - k_{2a}\,[\mathrm{E\text{-}I}] \qquad [6.39]$$

$$d[\mathrm{E\text{-}I\text{-}X}\cdot]/dt = k_{2a}\,[\mathrm{E\text{-}I}] - k_2\,[\mathrm{E\text{-}I}]\,[\mathrm{AH}] - k_3\,[\mathrm{E\text{-}I\text{-}X}\cdot] \qquad [6.40]$$

$$d[\mathrm{AOOH}]/dt = k_2\,[\mathrm{E\text{-}I\text{-}X}\cdot]\,[\mathrm{AH}] - k_1\,[\mathrm{E}]\,[\mathrm{AOOH}] \qquad [6.41]$$

Figure 6.9 shows how the rate parameters k_1, k_{2a}, and k_2 affect the shape of the kinetic trace according to the mechanism in Scheme 6.3. It was assumed that k_3 is the same as in the first model ($3 \times 10^{-3}\ s^{-1}$) . In all theoretical curves shown, except one, k_1 has a given value of $8 \times 10^7\ M^{-1}s^{-1}$ close to what was experimentally determined for 15-HPETE (14); as we showed, this is a good

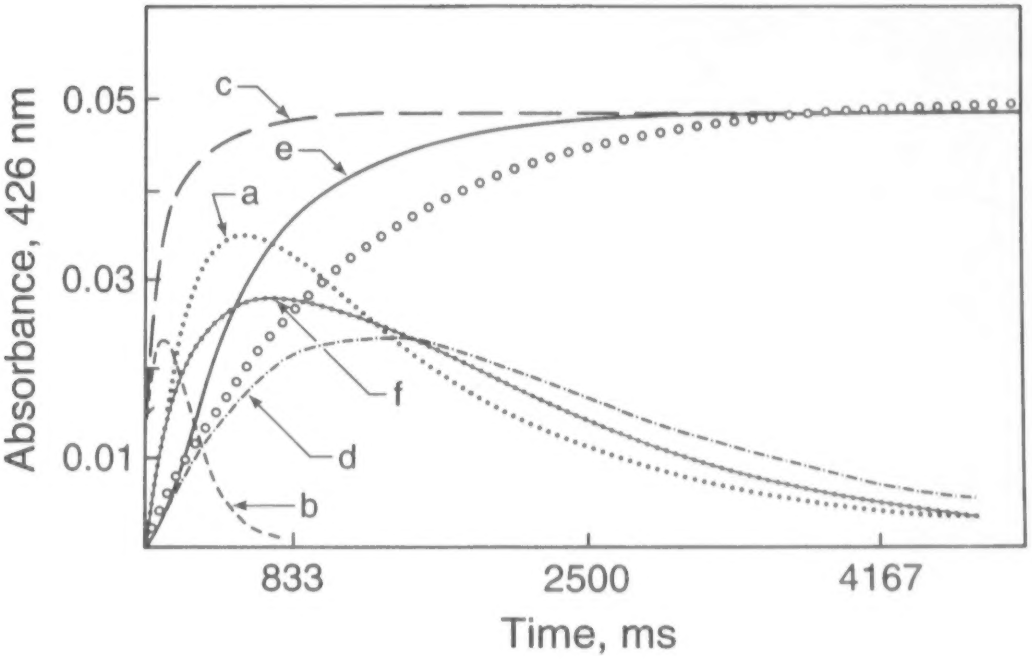

Figure 6.9. Simulated time courses according to the mechanism in Scheme 6.3. (o) exponential trace for experimental k_{obs} = 0.89 s^{-1}. **(a-f)**: theoretical curves for the same k_{obs} and initial conditions ($[AH]_o$ = 5.5 μM, $[A(O_2)OOH]_o$ = 0.25 μM, a nd $[E]_o$ = 0.25 μM); Combinations of the rate constants were as follows: **(a)** $k_1 = 8 \times 10^7$ M^{-1} s^{-1}, $k_2 = 1.2 \times 10^5$ $M^{-1}s^{-1}$, k_{2a} = 6.5 s^{-1} and $k_3 = 5 \times 10^{-1}$ s^{-1}.; **(b)** k_1, k_2 and k_3 as in (a), k_{2a} = 65 s^{-1} ; **(c)** k_1, k_{2a} and k_3 as in (b), $k_2 = 1 \times 10^2$ $M^{-1}s^{-1}$; **(d)** k_1, k_2 and k_3 as in (a) , k_{2a} = 1.2 s^{-1}; **(e)** k_1 and k_3 as in (a), k_{2a} = 2 s^{-1} and $k_2 = 1.2 \times 10^3$ $M^{-1}s^{-1}$; **(f)** $k_1 = 1.4 \times 10^7 M^{-1}$ s^{-1}, $k_2 = 1.2 \times 10^5$ $M^{-1}s^{-1}$, k_{2a} = 6. 5 s^{-1} and $k_3 = 5 \times 10^{-3}$ s^{-1};

estimate in the first cyclooxygenase model. The values for k_{2a} and k_2 were combined differently; under no conditions could a good fit with the experimental trace be obtained. Furthermore, for the values of k_1 and k_{2a} as determined experimentally at 1°C (4) and for the k_2 and k_3 with the most realistic values, both the formation and disapperance of the intermediate II would be faster than what was actually observed at 30°C (Figure 6.9, curve f).

6.4. DISCUSSION

6.4.1. Initial Conditions in the Pre-Steady State Kinetic Measurements

Since the detection of the tyrosyl radical (3) there has been controversy about its role in catalysis (18-20). It has been postulated that this enzyme species plays a key role in the initiation of the cyclooxygenase reaction by abstracting 13-*pro*-S hydrogen atom from 20:4. However, the kinetic mechanism of the cyclooxygenase reaction had never been studied in detail. Probably the most important reason is the instability of the enzyme intermediates which had limited the use of transient-state techniques. The problem is complicated by the fact that the tyrosyl radical has an identical Soret spectrum with compound II and that similar spectral changes have been observed in the reactions of PGH synthase with both hydroperoxides and 20:4 (1, 3, 4).

Another part of the problem originates in the sensitivity of 20:4 to autooxidation so that small amounts of contaminant hydroperoxide are always present in its solutions. Often very rigid purification procedures (21) did not completely remove the contaminant hydroperoxide from 20:4; there was always some trace amount present capable of initiating the cyclooxygenase reaction. In our preliminary experiments we found that the use of purified 20:4 makes the pre-steady state kinetic measurements very uncertain: the change in absorbance at 426 nm usually proceeded in more than a single step and this was accompanied with poor reproducibility. This happened because at low initial hydroperoxide concentration, an incomplete formation of compound I occurred followed by its conversion to more than one species , i.e., to compound II and compound I-tyrosyl radical. With unpurified 20:4 samples single exponential traces were obtained (Figure 6.2) with a satisfactory reproducibility which indicated that hydroperoxide concentration was constant and in excess of the enzyme so that the pseudo-first order conditions were satisfied for all three substrates, hydroperoxide, oxygen, and 20:4. For the series of the pre-steady state experiments with 20:4, the amount of oxygen was always constant (230 μM at 30°C) but the amount of contaminant hydroperoxide changed every time the amount of 20:4 was changed. In this situation the pre-steady state kinetics is affected by both hydroperoxide and 20:4 so that the kinetic data for 20:4 were subjected to the computer analysis .

The initial conditions in our pre-steady state experiments with 20:2 were adjusted in such a way that the effects of the fatty acid were fully separated from the effects of the hydroperoxide. We proceeded in an opposite direction from what used to be a common procedure: instead of purifying fatty acid from the traces of hydroperoxide we oxidized a series of 20:2 solutions for different periods of time so that we were able to vary the ratio of hydroperoxide and 20:2 in stock solutions and keep the hydroperoxide concentration constant and in excess in the later reaction with the enzyme. For this approach, 20:2 was chosen as an alternative to 20:4. Although it utilizes one molecule of oxygen (the oxygenase reaction) it is a reasonable good substrate for the enzyme and identical changes in the enzyme spectra were obtained to those with 20:4 (Figure 6.1), indicating that they probably share the same reaction mechanism. Furthermore, its hydroperoxide is a conjugated diene species that can be conveniently measured spectrophotometrically at 235 nm (6, 7). There is no more convenient method to measure the initial residues of the hydroperoxide in fatty acid solutions. In the past a polarographic procedure was used to measure the lipid peroxide concentrations by following the length of the lag phases in a cyanide-treated PGH synthase-cyclooxygenase reaction (22) but the latter method is less convenient than the spectrophotometric method.

6.4.2. The Inhibition of the Compound I Reaction with 20:4 by Indomethacin and 22:6

The effects of the cyclooxygenase inhibitors on the increase in absorbance at 426 nm (Figure 6.2) can be explained as follows: indomethacin and 22:6 prevent the reaction of compound I with 20:4 by competing with 20:4 for the same binding site. The two inhibitors have similar dissociation constants for the initial binding to the enzyme but the overall mechanisms of the inhibition are different (16). For 22:6, the inhibition is rapid and reversible while for indomethacin a rapid, reversible binding is followed by an irreversible first-order decay of an enzyme-inhibitor complex (16). This explains why the inhibition of the compound I reaction with 20:4 was more effective with indomethacin than with 22:6.

It has been demonstrated by e.p.r. studies (15, 23) that indomethacin and other cyclooxygenase inhibitors did not prevent the formation of tyrosyl radical signals although they inhibited the cyclooxygenase reaction. There are two distinct tyrosyl radical species detected during reactions with hydroperoxides or 20:4, a transient doublet which is replaced by a narrow singlet at longer times (3, 15, 24). A third tyrosyl radical signal has also been detected as a broad singlet in the presence of indomethacin or under conditions that enhance self-inactivation (15, 24). Its identity has been recently solved (25). The broad singlet is not a distinct tyrosyl radical species but a composite of the narrow singlet and the doublet originating from the same tyrosyl residue (25). The kinetics of the formation of doublet and singlet radical species did not correlate to the time course of 20:4 oxidation (24) or only the doublet is formed at a rate high enough to have catalytic competence in the cyclooxygenase reaction (15, 23). Since the doublet radical is transforming to the singlet radical and since both probably originate from the same, not from two different tyrosyl residues (25), these results are more in agreement with a role for the tyrosyl radical in oxidative auto-inactivation, not in catalysis. Our results are consistent with these findings. One might anticipate that if the tyrosyl radical were the species formed from compound I then indomethacin would have no effect on k_{obs} , which is not the case (Figure 6.2). Both indomethacin and 22:6 prevented the conversion of compound I to intermediate II. Thus, intermediate II probably is not compound I-tyrosyl radical. Rather it is either compound II or some unidentified compound I-protein radical having identical spectroscopic properties to those of compound II. In addition, Mn(III)-PGH synthase does not generate tyrosyl radicals but some unidentified protein radical and still is very active cyclooxygenase (24, 26). The authors of the tyrosyl radical hypothesis agree that some unidentified amino acid radical instead can play an active role in PGH synthase catalysis (18). Recently it was shown that mutation of tyrosine 385 with phenyl alanine (Y385F) abolished the cyclooxygenase activity but did not prevent the formation of the narrower singlet radical (27). The isotopic replacement with predeuterated tyrosine and phenyl alanine in Y385F mutant established that the singlet radical originates from an unknown tyrosine residue. This questioned the number and the identity of tyrosyl residues participating in catalysis and/or inactivation. Nonetheless, the entity presented in our model as E-I-X· (Schemes 6.1 and 6.2) can be either tyrosyl radical or any other protein radical which has the compound II-like heme structure.

6.4.3. Mechanism of the PGH Synthase Cyclooxygenase and Oxygenase Reactions

Our pre-steady state results and computer modelling revealed a unique mechanism for the oxidation of fatty acids (20:2 and 20:4) catalyzed by PGH synthase which lead to the formation of transient hydroperoxides (11-OOH-20:2 and PGG_2). According to our mechanism (Schemes 6.1 and 6.2) the heme group at the enzyme active site participates directly in all steps of the oxygenation reaction. The reaction of hydroperoxide with native enzyme leads to formation of the corresponding alcohol and compound I (the peroxidase reaction); compound I abstracts a hydrogen atom from the fatty acid producing compound II and fatty acid carbon radical (the initiation of the cyclo- and oxygenase reactions); the fatty acid carbon radical converts to the corresponding fatty acid hydroperoxyl radical after fast addition of one or two molecules of oxygen (the oxygenase and cyclooxygenase reactions). Compound II is a transient species under aerobic conditions which undergoes fast hydrogen atom abstraction at one protein residue by the hydroperoxyl radical and compound I-protein radical and corresponding hydroperoxide are formed (the completion of the cyclo- and oxygenase reactions). Overall, the conversion of the enzyme species is from $E \longrightarrow E\text{-}I \longrightarrow E\text{-}II \longrightarrow E\text{-}I\text{-}X\cdot$. The change in concentration of E, E-I, and E-I-X· species as a function of time is shown in Figure 6.6B. By using two different types of fatty acids we showed that the conversion of E-I is a function of fatty acid concentration a relationship typical for bimolecular reactions. We showed that E-II is also formed as a transient species under aerobic conditions. Since E-II has an identical spectra as E-I-X· no further distinctions between them were possible. We showed that the kinetic mechanism which includes the spontaneous $E\text{-}I \longrightarrow E\text{-}I\text{-}X\cdot$ reaction does not fit the experimentally observed kinetics (Figure 6.9).

The magnitudes of the rate constants for the reactions of lipid peroxides with native enzyme, k_1, are known from earlier transient state and steady-state studies (4, 12, 14, 17). Here for the first time we report the value for the k_1 obtained from a combination of the pre-steady state experiments and computer modelling. We showed that the rate constant k_1 is 4 $x10^7M^{-1}s^{-1}$ for the reaction of the native enzyme with HOO-20:2 and 8 $x10^7M^{-1}s^{-1}$ for the reaction with HOO-20:4. The value of $k_1 = 7.1 \times 10^7M^{-1}s^{-1}$ obtained under steady-state conditions with 15-HPETE, also hydroperoxide product of 20:4 (14), is the closest values to those predicted from our pre-steady state modelling. Thus, for the first catalytic step (the peroxidase reaction of the native enzyme) our pre-steady state model agrees with the previous studies. However, the pre-steady state kinetics disagrees with the previous hypothesis for the following catalytic step , i.e., the initiation of the cyclooxygenase reaction.

According to our model (Schemes 6.1 and 6.2), the reaction of compound I with fatty acid (E-II formation) appeared to be two orders of magnitude slower than the reaction of lipid peroxide with native enzyme (E-I formation). Our model predicts that the oxygen-related steps which occur after the reaction of compound I with fatty acid do not have an effect on the observed rate. The rate of E-I-X· return to the native enzyme E is a secondary reaction not significant on the time scales of our experiments. For the computer simulations we used an arbitrary low value of $k_3 = 3 \ x10^{-3} \ s^{-1}$. Thus, by modelling the cyclo- and oxygenase reactions we find out that we can apply more simple kinetic relationships for the 20:4 and 20:2 reactions with E- I. Initially we only knew that for the reaction with 20:2 the pseudo-first order conditions were satisfied. Without modelling we would not be able to find out that the same is true for the reaction with 20:4 and to see that oxygen concentration and the rate constants for the oxygen related steps do not affect the rate of the reaction of E-I with fatty acid. Finally, by modelling we showed the most probable sequence of events (Schemes 6.1 and 6.2) which does not agree with the tyrosyl radical hypothesis (Scheme 6.3).

Under pseudo-first order conditions, the observed reaction rate is the rate of the rate controlling step and the modelling showed that this is the reaction of compound I with fatty acid:

$$-d[E\text{-}I]/dt = k_2\,[AH]\,[E\text{-}I]$$

$$k_{obs} = k_2\,[AH]$$

Hence, the slopes of the curves in Figures. 6.4 and 6.7 represent the apparent second-order rate constant, k_2, for the reaction of 20:2 and 20:4 with PGH synthase compound I. Within experimental error, the k_2 values from the experiments ($1.2 \times 10^5\ M^{-1}s^{-1}$ for 20:4 and $2.5 \times 10^5\ M^{-1}s^{-1}$ for 20:2) and those obtained by the computer simulations are identical.

The spontaneous decay of compound I is insignificant in both 20:2 and 20:4 experiments since no significant intercepts were observed in the plots of k_{obs} versus [fatty acid] (Figures. 6.4 and 6.7). At least when the hydroperoxide concentration was constant and 10-fold in excess of the enzyme concentration, as in the experiments with 20: 2 (Figure 6.4), the intercept would be very obvious if the intramolecular electron transfer occurred to transform compound I to the compound I-tyrosyl radical. One can conclude that the spontaneous tyrosyl radical formation predicted from the kinetics of the reactions of native enzyme with hydroperoxides only (4) is not significant and does not occur simultaneously with the reaction of compound I with fatty acid. This could indicate that the fatty acid prevents the spontaneous decay of compound I to the tyrosyl radical (3,4).

6.5. CONCLUSIONS

Our pre-steady-state experiments and kinetic modelling reveal the identity of the oxidizing agent for the initiation of fatty acid oxygenation to be compound I, not compound I-tyrosyl radical. Rather the protein radical species does form simultaneously with hydroperoxide as a result of compound II reaction with the hydroperoxyl radical. We do not rule out the possibility that the protein radical can have a role in the events after the formation of the first molecule of hydroperoxide.

We provide kinetic evidence for a direct involvement of the heme prosthetic group in all steps of the oxygenation reaction (hydroperoxide formation). This indicates that the binding site of the fatty acid might be in a close proximity to the heme to allow the direct electron transfer from fatty acid to compound I intermediate, not remote from the heme as suggested in a model of the PGH synthase active site (28). This interpretation is consistent with the recent X-ray crystal structure of PGH synthase (29). Tyr^{385} is positioned near the edge of the heme and in the upper portion of the hydrophobic fatty acid binding site. Because of the low resolution of the enzyme-inhibitor complex the interaction of 20:4 with the active centre is not clear but a stereochemically build model of the 20:4 indicates that C-13 of 20:4 would be bound in the vicinity of Tyr^{385} and consequently in the vicinity of the heme. From our kinetic study it is very likely that fatty acid donates an electron directly to the porphyrin cation radical and a proton to a near basic amino acid residue of compound I.

Further structural models as for 20:4 binding are needed for $PGG_2\cdot$ binding in the fatty acid channel. This will be useful in the elucidation of the position and the orientation of the Tyr^{385} and other protein residues relative to the heme and the $PGG_2\cdot$ radical. The most prominent catalytic role of the enzyme is to stabilize the transient free radical intermediates and their transition states. The hydroperoxy radicals are probably situated in the fatty acid channel before the first hydroperoxide molecule is formed. We postulate that the tyrosyl radical is formed simultaneously with the first molecule of hydroperoxide. Thus all cyclooxygenase steps occur in the hydrophobic channel. The neutral hydroperoxide molecule is relocated to the peroxidase binding site after formation of the compound I-protein radical.

The kinetic mechanism proposed in this study will require further verification, particularly under anerobic conditions where the reaction of E-I + AH ⟶ E-II can be isolated from the oxygen-related steps. It will be also important to see whether the classical electron donors promote or inhibit the compound I reaction with fatty acid or have no effect.

The question of the rate controlling step in the overall cyclooxygenase reaction still remains open. The second order rate constant of the overall conversion of 20:4 to PGG_2 has been estimated to be $4 \times 10^4 M^{-1}s^{-1}$ (30) which is more than one order of magnitude lower than k_2. This suggests that step(s) after formation of the intermediate E-I-X·, are the most plausible candidates for the rate controlling step in the overall reaction. It has been shown before (12), and in our preliminary experiments, that the return of the intermediate II to the native enzyme coincides with the rate of the oxygen consumption in a PGH synthase-cyclooxygenase reaction.

Similar results were obtained with Mn(III)-PGH synthase (7). It was suggested that the formation of the Mn (IV) oxidized state which relates to Fe(IV) oxidized state in E-I-X· and/or the E-II, preceded or coincided to the hydroperoxide HOO-20:2 formation. This indicates that Fe(IV) species, E-I-X· and/or E-II, can be catalytically competent intermediates for the rate controlling step in the overall oxygenation. Mn(V) species, which oxidized state corresponds to Fe(V) state of E-I, was not detected.

E-I-X· could be a separate and distinct oxidizing agent from E-I and E-II but its role is still uncertain. In this study we showed that the compound I reaction with 20:4 and 20:2 is not complicated by the enzyme inactivation in a broad range (one order of magnitude) of 20:4 and 20:2 concentrations. However, the disappearance of intermediate II after the steady-state has been reached is accompanied by an extensive enzyme bleaching (Figure 6.2). Even though the reaction of intermediate II with fatty acids is slower and more convenient for kinetic measurements than the compound I reaction with fatty acids, the interpretation of the kinetic results is more challenging. Intermediate II appears to be the start of the pathway of auto-inactivation. This pathway has not yet been studied in detail but is important for an understanding the overall oxygenation mechanism.

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CHAPTER SEVEN

GENERAL DISCUSSION

Work on a catalytic mechanism of prostaglandin endoperoxide synthase (PGH synthase)* has been presented in this thesis. Several types of reactions were investigated: the peroxidase, the cyclooxygenase, the oxygenase and combined peroxidase-cyclooxygenase and peroxidase-oxygenase reactions. Suitable kinetic methods were developed for studying the PGH synthase kinetics under pre-steady-state and steady state conditions and when it was appropriate, the experimental data were combined with computer simulations in the final analysis.

The work began with the steady-state study of PGH synthase-peroxidase reaction of a series of cinnamic acid derivatives, *p*-coumaric, caffeic and ferulic acids. This was followed by an attempt to establish the relationship between the peroxidase and the cyclooxygenase functions of PGH synthase by utilizing ferulic acid and 20:4 as reducing substrates. The stoichiometry between the oxygen and the 20:2 oxygenation product was also established and a mechanism for the oxygenase reaction was proposed. The peroxidase versus the oxygenase function of PGH synthase was studied by utilizing Trolox C as a peroxidase substrate and 20:2 as an oxygenase substrate. Finally, the reaction of compound I with 20:2 and 20:4 was examined under pre-steady state conditions and a novel catalytic mechanism for the oxygenase and the cyclooxygenase reactions was revealed.

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* Abbreviations: PGH synthase, prostaglandin endoperoxide synthase; 20:4, arachidonic acid; 20:2, 11,14-eicosadienoic acid; DDC, diethyldithiocarbamate.

The cinnamic acid derivatives, *p*-coumaric, ferulic and caffeic acids, originate from the same metabolic pathway in higher plants and are ubiquitous in food products (1-3). They are all chain-breaking antioxidants (1,4) and reductants of the intracellular iron (5). They provide inhibition of the lipid peroxidation (4,6) and act as anti-cancer agents (7). Ferulic acid exhibits strong anti-inflammatory properties (8) and caffeic acid affects leukotriene biosynthesis by inhibiting the lipoxygenase catalyzed oxygenation of 20:4. These types of biological activity indicated that the cinnamic acid derivatives may also affect prostaglandin biosynthesis. This work has established that the cinnamic acid derivatives stimulate the prostaglandin biosynthesis by acting as reducing substrates of PGH synthase peroxidase.

The following evidence supports a mechanism in which *p*-coumaric and ferulic acids serves as reducing substrates for the PGH synthase-peroxidase through two one-electron oxidation steps: (i) the oxidation products of *p*-coumaric and ferulic acids are the same in the PGH synthase-hydrogen peroxide and the horseradish peroxidase-hydrogen peroxide systems, (ii) an identical steady-state enzyme intermediate (compound II) is present in both systems, and (iii) *p*-coumaric and ferulic acids stimulate the 20:4 oxygenation catalyzed by PGH synthase.

Previous steady-state kinetic studies on PGH synthase had serious limitations: techniques that had been used were inadequate and the interpretation of experimental results was incorrect (12,13). PGH synthase is a very sensitive enzyme and the kinetics is complicated by a high loss of activity. Because of that, the conventional spectroscopic methods (12,13) were too slow to produce accurate data. We have introduced a system for stopped-flow steady-state studies for accurate measurements of initial rates at short times when the enzyme inactivation was insignificant.

The earlier steady-state results were inaccurately interpreted in the manner of reversible Michaelis-Menten kinetics (13):

$$E + H_2O_2 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\text{-}I \xrightarrow{k'\,RH} E \qquad [7.1]$$

This mechanism is inappropriate because compound I (E-I) is not a real enzyme-substrate complex, but a distinct enzyme intermediate and because the second intermediate detected under steady-state conditions, compound II is not included in the reaction cycle.

Classical peroxidases obey the ping-pong kinetics that includes three irreversible steps (14):

$$E + H_2O_2 \xrightarrow{k_1} E\text{-}I + H_2O \qquad [7.2]$$

$$E\text{-}I + RH \xrightarrow{k_2} E\text{-}II + R\cdot \qquad [7.3]$$

$$E\text{-}II + RH \xrightarrow{k_3} E + R\cdot \qquad [7.4]$$

One molecule of the reducing substrate RH does not transfer two electrons in a single step (eq 7.1) but two molecules of RH transfer two electrons in the two one-electron steps (eqs 7.3. and 7.4).

Both mechanisms exhibit a saturation behavior and at a fixed [RH] a relationship between the reaction rate per total enzyme concentration, $v/[E]_{tot}$, versus the $[H_2O_2]$ exists as follows:

$$v/[E]_{tot} = A\,[H_2O_2]\,/\,(B + [H_2O_2]) \qquad [7.5]$$

Consequently, distinction between the two mechanisms is not possible from the primary plots of v/$[E]_{tot}$ versus $[H_2O_2]$. We showed that the best criteria to differentiate between the two mechanisms was to look at the secondary plots and the ratio of A/B. The ratio of A/B has different meaning in the two mechanisms. In the classical Michaelis-Menten mechanism, the ratio of A/B is a hyperbolic function of [RH]. For the irreversible ping-pong mechanism the ratio of A/B is a second order rate constant k_1. The experimental results were consistent with the irreversible ping-pong mechanism for both *p*-coumaric and ferulic acids as a reducing substrate. The second-order rate constants for the reaction of native enzyme with hydrogen peroxide (k_1 =4.6-6.4x10^5 $M^{-1}s^{-1}$) and for the reaction of compound II with *p*-coumaric acid (k_3 = 1.3 x10^6 $M^{-1}s^{-1}$) and ferulic acid (k_3 = 2.8 x 10^6 $M^{-1}s^{-1}$) were obtained. The value for the rate constant k_1 is 5-6 times higher than the value obtained by classical methods (13). The rate constant for the reaction of *p*-coumaric acid and PGH synthase compound II, k_3, is comparable to the value obtained by fast techniques for the reaction with horseradish peroxidase compound II (15).

The limitation of the steady-state method is that the rate constant for the reaction of compound I with reducing substrate, k_2, cannot be determined. However, the transient-state method does not provide information about this step either because of an extreme instability of compound I. For this particularly unstable enzyme the steady-state kinetics showed some advantages over the transient state kinetics. A broader range of experimental conditions can be applied and a lower amount (nM) of enzyme can be utilized. Deviations from the general peroxidatic mechanism, such as the inhibition of the reaction of hydrogen peroxide and native enzyme, as in the case of caffeic acid reaction, has been easily recognized by using the multiple-turnover conditions in the steady-state experiments. This behavior of the reducing substrate would not be so apparent in transient-state experiments with compound I or compound II.

Although a member of the same group of compounds, caffeic acid exerts a different peroxidatic mechanism from *p*-coumaric and ferulic acids. Caffeic acid is the strongest reducing agent in the series (16) but exhibited the lowest stimulation of the cyclooxygenase activity. The discrepancy between the two properties is explained with a specific mechanism that includes the formation of an inhibitory complex (E-RH) of caffeic acid (RH) with the native enzyme (E) followed by a three-step irreversible ping-pong peroxidation:

$$E + RH \overset{K_D}{\rightleftharpoons} E\text{-}RH \qquad [7.6]$$

$$E + H_2O_2 \xrightarrow{k_1} E\text{-}I + H_2O \qquad [7.7]$$

$$E\text{-}I + RH \xrightarrow{k_2} E\text{-}II + R\cdot \qquad [7.8]$$

$$E\text{-}II + RH \xrightarrow{k_3} E + R\cdot \qquad [7.9]$$

The above mechanism also shows the saturation kinetics:

$$v/[E]_{tot} = k_{cat}\,[H_2O_2] / (K_m + [H_2O_2]) \qquad [7.10]$$

The secondary plots are:

$$K_m/k_{cat} = 1/2k_1 + (1/2k_1\,K_D)\,[RH] \qquad [7.11]$$

$$k_{cat} = k_3\,[RH] \qquad [7.12]$$

Applying eqs. 7.11 and 7.12, the rate constants $k_1 = 5.7 \times 10^5\ M^{-1}s^{-1}$ and $k_3 = 1.3 \times 10^6\ M^{-1}s^{-1}$ were obtained.

Similar k_1 values in all three experiments indicate that the experimental conditions and reaction mechanisms were correct; hydrogen peroxide was used as the second substrate in all experiments. The rate constant k_3 for caffeic acid is the same as for *p*-coumaric acid and twice as low that for ferulic acid. This indicates that the second substituent (OCH_3 and OH) introduced in the *m*-position in the *p*-hydroxy cinnamic acid (*p*-coumaric acid) molecule does not significantly affect the rate of the reaction of these compounds with PGH synthase compound II. However the following characteristic is not observed with the monophenols *p*-coumaric and ferulic acids but is observed with the diphenol caffeic acid. Caffeic acid competitively inhibits ($K_D = 35\ \mu M$) the hydrogen peroxide reaction with native enzyme. The inhibition does not involve the coordination to heme iron nor the reduction of Fe^{III} of the heme. In case of the lipoxygenase inhibition, caffeic acid reduces non-heme Fe^{III} to Fe^{II} (11). Since no significant changes in the enzyme Soret spectra were obtained, the caffeic acid binding to PGH synthase probably includes hydrophobic interactions with protein residues situated near or at the hydroperoxide binding site.

Kinetic and spectral analysis disproves the previous postulate (17) that ferulic acid is demethylated by PGH synthase to caffeic acid in a reaction typical to cytochrome-P450. Ferulic acid is a typical peroxidase substrate for PGH synthase and the ferulic acid-product spectrum resembles the product spectrum obtained in the horseradish peroxidase catalyzed reaction. The ferulic acid product obtained in the horseradish peroxidase reaction is a dimer the structure of which has been unambiguously confirmed by mass spectrometry (17). In addition, caffeic acid is a good substrate for PGH synthase and consequently cannot be the stable product formed by the demethylation of ferulic acid.

The study of the caffeic acid binding addresses new questions with regard to the specificity and cooperativity in the PGH synthase-peroxidase catalysis. In diluted enzyme solutions, caffeic acid binding is diminished and the enzyme exhibits higher peroxidase activity. We attributed this behavior to a more intensive dissociation of the enzyme dimer in diluted solutions and to a higher enzymatic activity of two monomers than one dimer. It is known that some conformational changes could be involved in PGH synthase catalysis (18,19). A negative allosteric interaction between the two enzyme subunits has been suggested to cause lower than expected (1 mol heme/1 mol of subunit) stoichiometry of the heme (18,19). Based on the x-ray crystal structure, it is proposed that the membrane-bound enzyme is an active dimer (20) but why it is important that two molecules of 20:4 need to be released from the membranes and oxidized simultaneously is not apparent at the present.

A practical conclusion which arises from the study with cinnamic acid derivatives is that similar compounds capable of reducing lipoxygenase iron cannot be very selective inhibitors of leucotriene biosynthesis. These compounds could also reduce the higher oxidation states of PGH synthase and thus stimulate prostaglandin biosynthesis. However, reducing agents having different affinity for lipoxygenase and PGH synthase can be developed. Potentially they may be used to regulate the leucotriene/prostaglandin ratio when necessary, particularly under certain pathological conditions.

The cyclooxygenase-peroxidase function of PGH synthase was studied by comparing the initial rates of reaction of a cyclooxygenase substrate, arachidonic acid, and a peroxidase reducing substrate, ferulic acid, in mixtures of the two substrates. For both an equimolar ratio of arachidonic and ferulic acids and ferulic acid in excess of arachidonic acid a stimulation of the cyclooxygenase reaction is observed. A striking feature of our results is that PGH synthase catalyses oxidation of the two substrates in a constant and fixed molar ratio of ferulic acid to arachidonic acid of 2:1, despite widely different starting concentrations. If arachidonic acid is in excess of ferulic acid, enzyme inactivation occurs. The results are explained by an interconnected cyclooxygenase-peroxidase unbranched free-radical mechanism in which arachidonic acid (AH) reacts either with the ferryl oxygen or porphyrin π cation radical part of the conventional peroxidase compound I (E-I) and ferulic acid (RH) with compound II (E-II). The ferulic acid also acts as a hydrogen atom donor to the hydroperoxyl radical of PGG_2, $A(O_2)OO\cdot$, or alternatively to the tyrosyl radical (E-I-Tyr·) providing protection from inactivation:

$$
\begin{array}{rcll}
\text{E} + A(O_2)OOH & \longrightarrow & \text{E-I} + A(O_2)OH & [7.13] \\
\text{E-I} + AH & \longrightarrow & \text{E-II} + A\cdot & [7.14] \\
A\cdot + 2O_2 & \longrightarrow & A(O_2)OO\cdot & [7.15] \\
\text{E-II} + A(O_2)OO\cdot & \longrightarrow & \text{E-I-Tyr}\cdot + A(O_2)OOH & [7.16] \\
\text{E-I-Tyr}\cdot + RH & \longrightarrow & \text{E-II} + R\cdot & [7.17] \\
\text{E-II} + RH & \longrightarrow & \text{E} + R\cdot & [7.18] \\
\hline
AH + 2O_2 + 2\,RH & = & A(O_2)OH + 2R\cdot & [7.19]
\end{array}
$$

Our data provides strong evidence against the branched-chain free radical mechanism (21,22), in which a cyclooxygenase cycle, once started, can operate with no connection to the peroxidase cycle. A consequence of the unbranched-chain mechanism (eqs 7.13-7.19) is that the hydroperoxide PGG_2 is consumed stoichiometrically during the cycle. A support for this mechanism is found in the product analysis studies (23-25) where, in the presence of a peroxidase reducing substrate (phenol) the exclusive product of 20:4 oxidation is PGH_2 not PGG_2.

Our proposal (eqs 7.13-7.19) is restricted to the peroxidase-cyclooxygenase reaction. Without a reducing substrate present both PGH_2 and

PGG_2 were detected (23-25). This implies that the spontaneous cyclooxygenase reaction could proceed partially through the same pathway i.e., the first four steps could be the same as in the peroxidase-cyclooxygenase reaction to form the hydroperoxide but a mechanism including the branched-chain free radical mechanism could not be excluded after the first molecule of hydroperoxide was formed. The spontaneous reaction is accompanied with an intensive enzyme bleaching so that it is difficult to distinguish between the real catalytic reaction and the inactivation.

We recognize that the measurements of the rates of PGG_2 and PGH_2 formation can be very useful in distinguishing between the newly proposed (eqs. 7.13-7.19) and the previously published (21,22) catalytic mechanism. However we were unable to distinguish PGG_2 and PGH_2 formation. 20:4, PGG_2. and PGH_2 are colorless compounds and spectral kinetic methods cannot be applied for their determination. The lack of more information about the catalytic mechanism of PGH synthase in the past partly can be attributed to this problem. To build more evidence for the proposed unbranched chain mechanism we found that the study of the oxygenation reaction of 20:2 can be a useful alternative for the cyclooxygenation reaction of 20:4. 20:2 is oxidized by PGH synthase in a manner similar to 20:4, except that one molecule of oxygen is incorporated at C_{11} to form 11-hydroperoxy and 11-hydroxy fatty acid derivatives (26,27). The oxygenation products have conjugated double bonds which strongly absorbs UV light (28). This property allowed us to measure the rates of formation of the fatty acid oxygenation products and compare them with the rates of oxygen consumption under the same experimental conditions.

It is fortunate that 20:2 is a good substrate for lipoxygenase (26,28). Lipoxygenase catalyses the insertion of one molecule of oxygen at C_{15} of 20:2 forming a 15-hydroperoxy isomer, having the same number of conjugated double bonds as the 11-hydroperoxy isomer (26). The reaction mechanism of lipoxygenase is very well established and a 1:1 molar ratio between oxygen and the hydroperoxide is expected during the fatty acid oxygenation (29). Because of that, the lipoxygenase reaction was the most adequate reaction to be used as a reference in the investigation of the 20:2 oxygenation by PGH synthase.

The expected 1:1 molar ratio of the conjugated diene and oxygen for the lipoxygenase reaction and an unexpected 2:1 molar ratio for the PGH synthase reaction were obtained. The 2:1 stoichiometry of PGH synthase oxygenase reaction is explained by the existence of the two interrelated routes (eqs 7.20-7.25), each of which results in the formation of the conjugated diene hydroxy derivative of the acid (AOH):

$$\text{E} + \text{AOOH} \longrightarrow \text{E-I} + \text{AOH} \qquad [7.20]$$

$$\text{E-I} + \text{AH} \longrightarrow \text{E-II} + \text{A}\cdot \qquad [7.21]$$

$$\text{A}\cdot + \text{O}_2 \longrightarrow \text{AOO}\cdot \qquad [7.22]$$

$$\text{E-II} + \text{AOO}\cdot \longrightarrow \text{E-I-Tyr}\cdot + \text{AOOH} \qquad [7.23]$$

$$\text{E-I-Tyr}\cdot + \text{AH} \longrightarrow \text{E-II} + \text{AOH} \qquad [7.24]$$

$$2\,\text{AH} + \text{O}_2 = 2\,\text{AOH} \qquad [7.25]$$

One route, initiated by hydrogen atom abstraction from the acid (AH) by compound I (E-I), results in the formation of the conjugated diene hydroperoxy derivative (AOOH). The latter is converted to the hydroxy derivative in regenerating compound I from the native enzyme (E). The other route involves direct oxygen atom insertion into the acid by the tyrosyl radical form of compound I (E-I-Tyr·).

Now we will compare the steady-state results for the cyclooxygenase and the oxygenase reactions. We predicted that ferulic acid provides electrons to either $PGG_2\cdot$ or compound I-tyrosyl radical and compound II and thus stimulates the cyclooxygenase reaction and prevents the inactivation. We obtained kinetic evidence that the spontaneous oxygenase reaction operates through a specific mechanism which includes an active role of the compound I-tyrosyl radical in the reaction cycle. We lacked any evidence that the compound I-tyrosyl radical subtracts the hydrogen atom from 20:4 in the spontaneous cyclooxygenase reaction so that we do not know whether the cyclooxygenase reaction follows a different mechanism from the oxygenase reaction in the absence of peroxidase reducing substrates.

The Trolox C oxidation product is the same in the PGH synthase and horseradish peroxidase reactions and we initially assumed that Trolox C plays the same role in the oxygenase reaction as the ferulic acid plays in the cyclooxygenase reaction. However, we do not know how ferulic acid affects the stoichiometry between oxygen and 20:4 oxygenation products of the spontaneous cyclooxygenase reaction. We provide evidence that Trolox C did not affect the stoichiometry of the oxygenase reaction so that it did not react with the compound I-tyrosyl radical. This indicated that at least two compound I-protein radicals can be formed during the oxygenase reaction, one involved in catalysis, the other in inactivation. The existence of more than one protein radical has been reported for myoglobin and cytochrome c peroxidase (30-32). For PGH synthase the existence of two tyrosyl radicals has been recently confirmed by site-directed mutagenesis studies (33). A mutation of Tyr^{385} with Phe^{385} did not prevent the formation of a wide singlet signal from the tyrosyl radical. This second tyrosyl radical was associated with enzyme inactivation. It is not known whether a reducing substrate can prevent the appearance of the mutant radical. Such findings would be more direct proof for the role of Trolox C to reduce the amount of the unproductive enzyme radical as is proposed in this study. It is known that phenol prevents the formation of the doublet tyrosyl radical in the wild type PGH synthase (34). It is believed that the doublet radical originates from Tyr^{385} and therefore actively participates in catalysis (35,36). However none of these studies prove that the doublet tyrosyl radical is an obligatory intermediate in cyclooxygenase catalysis, and it remains possible that this radical is also formed during the oxidative inactivation.

By the pre-steady state kinetic study of the oxygenase reaction with 20:2 and the cyclooxygenase reaction with 20:4 we exclude the tyrosyl radical as a species that initiates the oxygenation of fatty acids. The intra-molecular electron transfer from a tyrosine residue to the porphyrin π cation radical of compound I

$$\text{E-I} \longrightarrow \text{E-I-Tyr}\cdot \qquad [7.26]$$

is a key step in the branched-chain tyrosyl radical mechanism (21). The formation of the tyrosyl radical is a unimolecular reaction independent on fatty acid concentration. In the next step, the tyrosyl radical formed abstracts the hydrogen atom from fatty acid and thus initiates the oxygenation.

Our steady-state models suggested that compound I abstracts a hydrogen atom from the fatty acid

$$\text{E-I} + \text{AH} \longrightarrow \text{E-II} + \text{A}\cdot \qquad [7.27]$$

This reaction is a bimolecular reaction where rate is a function of fatty acid concentration.

The two possibilities were distinguished under pre-steady state conditions by following the rate of disappearance of compound I (the compound I-tyrosyl radical or compound II formation) as a function of fatty acid concentration. From combined experiments and numerical simulations it is found that the rate of disappearance of compound I is a linear function of fatty acid concentration and thus the existence of the electron (hydrogen atom) transfer from fatty acid to compound I (eq 7.27) confirmed in the most direct way.

The results of the pre-steady-state kinetics and modelling are consistent with the steady state results. An identical catalytic pathway which leads to the hydroperoxide formation in both the cyclooxygenase (7.13-7.16) and the oxygenase (eqs 7.20-7.23) reactions were revealed as follows:

$$\mathrm{E + AOOH \longrightarrow E\text{-}I + AOH} \qquad [7.20]$$

$$\mathrm{E\text{-}I + AH \longrightarrow E\text{-}II + A\cdot} \qquad [7.21]$$

$$\mathrm{A\cdot + O_2 \longrightarrow AOO\cdot} \qquad [7.22]$$

$$\mathrm{E\text{-}II + AOO\cdot \longrightarrow E\text{-}I\text{-}Tyr\cdot + AOOH} \qquad [7.23]$$

In the first step a fatty acid hydroperoxide reacts with the native enzyme to form compound I and the fatty acid hydroxide. In the second step fatty acid reduces compound I to compound II and the fatty acid carbon radical is formed. This is followed by two fast steps: (i) the addition of either one molecule of oxygen (the oxygenase reaction) or two molecules of oxygen (the cyclooxygenase reaction, not shown) to the fatty acid carbon radical to form the corresponding hydroperoxyl radical, and (ii) the reaction of the hydroperoxyl radical with compound II to form the fatty acid hydroperoxide and a compound I-tyrosyl radical.

The implication of the above mechanism is that the tyrosyl radical formation is not an initiation step in the oxygenation of fatty acid but its formation is a consequence of the overall oxygenation of the fatty acid to the corresponding hydroperoxide.

The simulated second order rate constants for the conversion of native enzyme to compound I with 20:4 or 20:2 hydroperoxides as a substrate are 8 x 10^7 and 4 x 10^7 $M^{-1}s^{-1}$ respectively and in agreement with the previous findings (37,38). The simulated and experimentally obtained second order rate constants for the conversion of compound I to compound II with arachidonic and eicosadienoic acids as a substrate, k_2, are 1.2 x10^5 and 3 x 10^5 $M^{-1}s^{-1}$ respectively. They were obtained for the first time in this study.

SUGGESTIONS FOR FURTHER WORK

The important conclusion from our pre-steady state experiments is that because the oxygen-related steps do not affect the rate of the reaction of compound I with fatty acid a simplified equation $-d[E\text{-}I]/dt = k_{obs} [E\text{-}I]$, with $k_{obs} = k_2 [AH]$ can be applied for the determination of the second order rate constant k_2. The pre-steady state method could be a convenient way to study a structure-activity relationship for the reaction of a homologous series of fatty acids with compound I. This study could contribute to a better understanding of the role of compound I to initiate the oxygenation and to find what kinds of steric and electronic restrictions are imposed on the fatty acid binding and oxygenation.

The hypothesis that the peroxidase reducing substrate does not compete with fatty acid for the reaction with compound I in a peroxidase-cycloxygenase reaction could be verified by following the compound I reaction with fatty acid in the presence and absence of some peroxidase reducing substrate.

A similar approach could be undertaken to study the reaction of compound I-protein radical and compound II with fatty acids. We indicated with the modelling study that the formation of the compound I-protein radical is a branched point in the cyclooxygenase reaction. The differences between the reaction mechanisms for the spontaneous and the stimulated cyclooxygenase reaction were more apparent after the first molecule of hydroperoxide and the compound I-protein radical were formed. In the presence of a fatty acid, the compound I-protein radical is converted to the native enzyme at least one order of magnitude slower than by how much compound I disappears to compound II . Because the compound I-protein radical disappearance is slow, the reaction is convenient for the stopped flow measurements but the process is accompanied with an extensive heme bleaching. We did some preliminary experiments that indicated that phenol could prevent the bleaching with 20:4 and even regenerate the heme to some extent during the same reaction. It is vital to understand these processes. The inactivation by hydroperoxides shows an unpredictable pathway and good kinetic results could not be obtained in the absence of reducing substrates. However, competitive experiments that involve a hydroperoxide, a peroxidase reducing substrate and a fatty acid could be useful to obtain more information whether the fatty acid reacts with compound I-protein radical or if it is a classical reaction of compound II and fatty acid accompanied with the compound I-protein radical inactivation reaction.

Additional mechanistic information can also be obtained by studying the reaction kinetics of the Mn^{III}-PGH synthase. This modified enzyme is interesting in many ways and almost no kinetic data is available at the present. It will be important to see what stoichiometry exists between a peroxidase reducing substrate and a fatty acid, or oxygen and an oxygenation product, if it was obtained with the enzyme modified in such a way that is a very active cyclooxygenase and a very poor peroxidase (34,39). In addition, no tyrosyl radicals but some unknown protein radicals were detected during the reactions of the Mn^{III} enzyme with hydroperoxides (34). This also indicate that some differences in the catalytic mechanisms of the Mn^{III} and Fe^{III} enzymes exist. Maybe similarly interesting would be the kinetic and mechanistic studies with the mutant enzymes, particularly those predominantly exhibiting the cyclooxygenase or the peroxidase activity (33).

Other areas of the PGH synthase catalysis also remain to be investigated. Very little is known about the PGH synthase active site residues that participate in the catalysis. An acid dissociation constant with pK_a of 4.2 was discovered with the study of the cyanide binding to the native enzyme (40) but the identity of the group responsible for this ionization is not known. Now that the crystal structure of PGH synthase is available (20), the pH dependence studies become crucial. Possibly the least complicated and the best way to start would be the pH dependence study of the reaction of the native enzyme with hydroperoxides (the compound I formation).

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APPENDIX A

A.1. Derivation of Steady State Rate Law for the Reaction of *p*-Coumaric Acid and PGH Synthase. Mechanisms are defined in Figure 2. 7.

A.1.1. Mechanism I

Rate:

$$\frac{d[\text{Compound I}]}{d\,t} = k_1\ [E]\,[H_2O_2\,] - k_2\ [\text{Compound I}]\,[CA] = 0 \qquad [A.1]$$

$$\frac{d[\text{Compound II}]}{d\,t} = k_2[\text{Compound I}]\,[CA] - k_3\,[\text{Compound II}]\,[CA] = 0 \qquad [A.2]$$

Mass balance:

$$[E]_{tot} = [\text{Compound I}] + [\text{Compound II}] + [E] \qquad [A.3]$$

$$[E]_{tot} = (\ \frac{k_3}{k_2} + \frac{k_3[CA]}{k_1[H_2O_2]} + 1)\ [\text{Compound II}] \qquad [A.4]$$

Initial rate expression:

$$\frac{-\ d[H_2O_2]}{dt} = \frac{-1}{2}\ \frac{d[CA]}{dt} = k_3\,[CA]\,[\text{Compound II}] =$$

$$= \frac{k_1k_2k_3\,[CA]\,[H_2O_2]\,[E]_{ot}}{k_2k_3\ [CA] + k_1\ (k_2 + k_3)\,[H_2O_2]} \qquad [A.5]$$

Rearranging equation [A.5]:

$$\frac{\frac{-\ d\,[H_2O_2]}{dt}}{[E]_{tot}} = \frac{\frac{k_2\,k_3}{(k_2 + k_3)}\ [CA]\,[H_2O_2]}{\frac{k_2k_3}{k_1(k_2 + k_3)}\ [CA] + [H_2O_2]} = \frac{A\ [H_2O_2]}{B + [H_2O_2]} \qquad [A.6]$$

$$A = \frac{k_2k_3\,[CA]}{(k_2 + k_3)}\ ;\ B = \frac{k_2k_3\,[CA]}{k_1(k_2 + k_3)}\ ;\ \frac{A}{B} = k_1 \qquad [A.7]$$

The ratio A/B is constant independent of the CA concentration.

A.1.2 . Mechanism II

Rate:

$$\frac{d[\text{Compound I}]}{d\,t} = k_1[E][H_2O_2] - (k_{-1} + k_2[CA])[\text{Compound I}]=0 \qquad [A.8]$$

$$\frac{d[\text{Compound II}]}{dt} = k_2\,[CA]\,[\text{Compound I}] - k_3\,[CA]\,[\text{Compound II}] =0 \qquad [A.9]$$

Mass balance:

$$[E]_{tot} = [E] + [\text{Compound I}] + [\text{Compound II}] \qquad [A.10]$$

$$[E]_{tot} = \left\{ \frac{k_3(k_{-1} + k_2\,[CA])}{k_1k_2\,[H_2O_2]} + \frac{k_3}{k_2} + 1 \right\} [\text{Compound II}] \qquad [A.11]$$

Initial rate expression:

$$\frac{-d[H_2O_2]}{dt} = \frac{-1}{2}\,\frac{d[CA]}{dt} = k_3\,[CA]\,[\text{Compound II}]$$

$$= \frac{k_1k_2k_3\,[CA]\,[H_2O_2]\,[E]_{tot}}{k_3(k_{-1}+ k_2\,[CA]) + k_1(k_3+ k_2)\,[H_2O_2]} \qquad [A.12]$$

Rearranging equation A.12:

$$\frac{\frac{-d[H_2O_2]}{dt}}{[E]_{tot}} = \frac{\frac{k_2k_3}{(k_2+ k_3)}\,[CA]\,[H_2O_2]}{\frac{k_3(k_{-1}+ k_2[CA])}{k_1(k_2+ k_3)} + [H_2O_2]} = \frac{A[H_2O_2]}{B + [H_2O_2]} \qquad [A.13]$$

$$A = \frac{k_2k_3\,[CA]}{(k_2+ k_3)}\ ;\ B = \frac{k_3\,(k_{-1}+ k_2\,[CA])}{k_1\,(k_2 +k_3)} \qquad [A.14]$$

$$\frac{A}{B} = \frac{k_1[CA]}{\frac{k_{-1}}{k_2} + [CA]} \qquad [A.15]$$

The ratio A/B is a hyperbolic function of the CA concentration.

A.1.3 . Mechanism III

Rate:

$$\frac{d[\text{Compound I}]}{dt} = k_1[H_2O_2]\,[E] - (k_1 + k'\,[CA])\,[\text{Compound I}] = 0 \qquad [A.16]$$

Mass balance:

$$[E]_{tot} = [E] + [\text{Compound I}] \qquad [A.17]$$

$$[E]_{tot} = \left(\frac{k_{-1} + k'\,[CA]}{k_1[H_2O_2]} + 1\right)[\text{Compound I}] \qquad [A.18]$$

Initial rate expression:

$$\frac{-d[H_2O_2]}{dt} = \frac{-d[CA]}{dt} = k'\,[CA][\text{Compound I}]$$

$$= \frac{k_1k'\,[CA]\,[H_2O_2]\,[E]_{tot}}{(k_{-1} + k'\,[CA]) + k_1[H_2O_2]} \qquad [A.19]$$

Rearranging equation A.19:

$$\frac{\frac{-d[H_2O_2]}{dt}}{[E]_{tot}} = \frac{k'\,[CA][H_2O_2]}{\frac{k_{-1} + k'\,[CA]}{k_1} + [H_2O_2]} = \frac{A\,[H_2O_2]}{B + [H_2O_2]} \qquad [A.20]$$

$$A = k'\,[CA]\,; \qquad B = \frac{k_{-1} + k'\,[CA]}{k_1}\,; \quad \frac{A}{B} = \frac{k_1[CA]}{\frac{k_{-1}}{k'} + [CA]} \qquad [A.21]$$

The ratio A/B is a hyperbolic function of the CA concentration.

APPENDIX A.2 Derivation of Steady State Rate Law for the Reaction of Caffeic Acid and H_2O_2 Catalyzed by PGH Synthase

According to the mechanism presented with eqs. 3.1-3.4:

Mass balance:

$$[\mathrm{PGHS}]_{tot} = [\mathrm{PGHS}] + [\mathrm{PGHS\text{-}CAF}] + [\mathrm{PGHS\text{-}I}] + [\mathrm{PGHS\text{-}II}] \qquad [\mathrm{A.16}]$$

Rate:

$$v = -d[\mathrm{CAF}]/dt = 2\, k_3\, [\mathrm{CAF}]\, [\mathrm{PGHS\text{-}II}] \qquad [\mathrm{A.17}]$$

Steady state conditions:

$$d[\mathrm{PGHS\text{-}I}]/dt = k_1\, [\mathrm{PGHS}]\, [H_2O_2] - k_2\, [\mathrm{PGHS\text{-}I}]\, [\mathrm{CAF}] = 0 \qquad [\mathrm{A.18}]$$

$$d[\mathrm{PGHS\text{-}II}]/dt = k_2\, [\mathrm{PGHS\text{-}I}]\, [\mathrm{CAF}] - k_3\, [\mathrm{PGHS\text{-}II}]\, [\mathrm{CAF}] = 0 \qquad [\mathrm{A.19}]$$

Reaction equilibrium:

$$K_D = \frac{[\mathrm{PGHS}]\, [\mathrm{CAF}]}{[\mathrm{PGHS\text{-}CAF}]} \qquad [\mathrm{A.20}]$$

$$[\mathrm{PGHS}]_{tot} = \left\{ \frac{k_3\, [\mathrm{CAF}]}{k_1\, [H_2O_2]} + \frac{k_3\, [\mathrm{CAF}]^2}{K_D\, k_1\, [H_2O_2]} + \frac{k_3}{k_2} + 1 \right\} [\mathrm{PGHS\text{-}II}] \qquad [\mathrm{A.21}]$$

$$\frac{-d[\mathrm{CAF}]/dt}{[\mathrm{PGHS}]_{tot}} = \frac{k_{cat}^{app}\, [H_2O_2]}{K_m^{app} + [H_2O_2]} \qquad [\mathrm{A.22}]$$

$$k_{cat}^{app} = \frac{2\, k_1\, k_2\, k_3\, [\mathrm{CAF}]}{k_1\, (k_2 + k_3)} \qquad [\mathrm{A.23}]$$

$$K_m^{app} = \frac{k_2 k_3\, [\mathrm{CAF}]\, (1 + [\mathrm{CAF}]/ K_D)}{k_1\, (k_2 + k_3)} \qquad [\mathrm{A.24}]$$

$$\frac{k_{cat}^{app}}{K_m^{app}} = \frac{2\, k_1}{1 + [\mathrm{CAF}]/K_D} \qquad [\mathrm{A.25}]$$